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EFFECTS OF INHALATION ANESTHETICS ON PRODUCTION OF KETONE BODIES IN RAT LIVER SLICES*

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(Received for publication, July 23, 1954)

Effects of inhalation anesthetics on respiration, carbohydrate metabolism, and acid-base balance of living substances have been studied *in vivo* already by many workers (*1–6*). Besides, there are similar investigations which were performed *in vitro* (*7*, *8*). However so far as the author knows, effects of inhalation anesthetics on metabolism of ketone bodies *in vitro* have not yet been studied. Moreover, while many fundamental researches concerning to ether anesthesia have been made, those of trichloroethylene, which had been used clinically since 1934 (*9*), are comparatively few. Therefore, out of various kinds of inhalation anesthetics, which are used at the surgical and obstetrical clinics, ethyl ether (E) and trichloroethylene (TCE) were chosen in order to study their effects on production of ketone bodies in rat liver slices and to compare them with those of epinephrine (EP) or CCl_4, administration groups.

EXPERIMENTAL

Materials

Test Animals—Female rats, weighing 100 to 150 g., were used. They were fed previously with standard diet (Oriental Pellet Diet MCS), and divided into five groups:

1) Unoperated control group: mere abstention from diet for 24 hours.
2) E anesthesia group: Upon 24 hours of abstention from diet, rats were put in desiccator, containing a lump of cotton soaked with E, and let to inhale E vapour until respiration stopped. It took generally 5 minutes.
3) TCE anesthesia group: Upon 24 hours abstention from diet, rats were put in desiccator into which TCE vapour was puffed. TCE vapour was

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generated in warmed test tube containing TCE. In this case, respiration accelerated in the course of anesthesia, and it was difficult to obtain deep anesthesia (*10*). Therefore, mark point was set at the stage, in which respiration of costal type could be solely observed. That stage will probably correspond to the plane 3 of 3rd stage of anesthesia (*11*).

4) CCl_4 administration group: rats, fasted for 16 hours, were administered intraperitoneally with 0.1 ml. of CCl_4 per 100 g. of body weight, and fasted another 2 hours.

All rats were sacrificed by decapitation, bled well, and slices were prepared according to the following procedure:

Preparation of Slices—The liver, kidney and brain were taken out immediately and bathed in ice-cold Krebs-Ringer phosphate buffer (*12*). Slices approximately 0.25–0.30 mm. thick, were made free hand and distributed into each independent vessels according to Table I. Since metabolism of liver slices was stated to be influenced by the concentration of potassium and calcium in the media (*13*), the same amount of potassium, in the form of KCl, was added to the media, in which no potassium butyrate was added previously.

TABLE I

Experimental Conditions

Main compartment	*ml.*	*ml.*	*ml.*	*ml.*
Krebs-Ringer phosphate buffer (pH 7.4)	3.0	3.0	3.0	3.0
H_2O	—	—	0.5	0.5
0.02 *M* KCl	—	0.5	—	—
Tissue slices	liver	liver	kidney	brain
(dry weight)	(10-20 mg.)		(8-10 mg.)	
Side arm				
0.02 *M* Potassium butyrate	0.5	—	—	—
Center well*				
10% KOH	0.2	0.2	0.2	0.2

Flasks were incubated in an atmosphere of 100 per cent oxygen in Warburg water bath at 37.5°, with constant shaking at the rate of 110–120 strokes per minute, for 60 minutes.

* Rolls of filter-paper were inserted.

At the end of experiment, the slices were removed from the vessels, washed in destilled water, dried at 105°, and were weighed. In order to prevent dissipation of narcotics into media out of narcotized tissues during shaking, certain amount of narcotics were added preliminarily in the media (*14*). In case of E, the amount added in the media was such value as to reach the final concentration equivalent to the blood concentration in man under deep anesthesia (*11*). The concentration, 2.45 mg./dl., was determined by Sugayama by measuring the amount of reduced $K_2Cr_2O_7$ (*15*). TCE and EP were added by 0.1 ml. and 1.5 ml. (0.01 per cent solution), respectively, per 100 ml. of media.

Air phases of flasks were replaced by 100 per cent oxygen, and the materials were incubated in Warburg water bath at 37.5° and given shaking of 110–120 strokes per minute. It took an hour from decapitation to the commencement of shaking.

Methods

Determination of O_2-uptake—After 10 minutes of shaking for the attainment of temperature equilibrium, readings of oxygen uptake were recorded every 15 minutes for the next one hour. The reading of the first 15 minutes was neglected, and the average oxygen uptake was calculated over the remaining readings. After the experiment the contents of the flasks were decanted into a graduated tube and deproteinized according to Lipsett (*16*) by $Ba(OH)_2-ZnSO_4$ method.

Determination of Total Ketone Bodies—This was carried out on the filtrate according to the method of Greenberg and Lester (*17*).

Determination of Butyric Acid—Remaining butyric acid was measured on the filtrate by slightly modified method of Klink (*18*) *i. e.*, aliquot part of the filtrate was made acid by adding H_2SO_4, and steam-distilled in order to remove Cl^- which interferes the procedures, with the modified apparatus of Pozzi-Escot (*19*). To the aliquot part of the destillate, Scott-Wilson's reagent was added, and preformed acetone was determined by measuring its turbidity. On the other hand, the other aliquot part of the destillate was mixed with H_2O_2 and iron sulphuric acid, and steamdistilled into a graduated small test tube containing Scott-Wilson's reagent. After shaking the test tube gently and left standing still for 10–15 minutes, its turbidity was measured by Hidachi's spectrophotometer, at the wave-length of 660 $m\mu$. Difference between this value and that of preformed acetone will correspond to the amount of acetone originated from butyric acid. Since the turbidity developed from one unit of butyric acid will correspond to that developed from 0.345 units of acetone, the amount of butyric acid was calculated by dividing that difference by 0.345. Standard curve was drawn according to the foot-note to Fig. 1.

Amount of butyric acid consumed was calculated by subtracting the remnants in the flasks from the amounts added prior to the experiment (10 μM).

Determination of Fat—Total fat in the original liver was determined by the method of Bragdon (*20*).

Determination of Carbohydrate—Total and free carbohydrate in the liver and kidney cortex were determined by the modified method of Yamamoto (*21*): Upon hydrolization in 3.3 g./dl. HCl, or in 30 g./dl. KOH, samples were neutralized by KOH or HCl using Congo red paper as the indicator, and deproteinized (*22*) by $Ba(OH)_2$-$ZnSO_4$. The filrate was treated by the method described in the original article, and

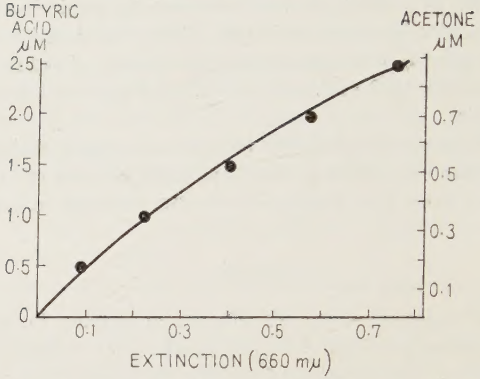

Fig. 1. Standard curve of butyric acid and acetone.

Mixture of H_2O_2, iron sulphuric acid, and the known amount of butyric acid or acetone, was steam-destilled into a graduated small test tube containing 3.0 ml. of Scott-Wilson's reagent, until the surface of the destillate reached at 5.0 ml. level. After shaking the test tube gently and left standing still for 10–15 minutes, its turbidity was measured by Hidachi spectrophotometer, at wave-length of 660 mμ. Thickness of cuvette, 10 mm.

finally spectrophotometric reading was conducted at the wave length of 520 mμ. Standard graph was a straight line, which pass the origin.

RESULTS AND DISCUSSION

I. Oxygen Uptake in Various Tissue Slices

Oxygen uptake in brain, kidney, and liver was shown in Tables II and III, and compared by group.

O_2-uptake in Brain and Kidney—As shown on Table II, average Q_{O_2} of brain and kidney cortex, in group of unoperated control, are −8.3 and −18.2, respectively; these figures are comparable with Enomoto's results of −7.2 and −15.8 obtained in rabbits (*8*). In group of E. anesthesia average oxygen uptake in brain decreases significantly to −6.6, which is comparable with −6.0 by Enomoto (*8*). These results agree in general with those obtained by other worker (7). Incidentally, there were significant decreases of oxygen uptake in brain in groups of TCE anesthesia and of CCl_4 administration. Mechanism of the latter decrease is hardly explained, and further experiments

TABLE II

Endogenous Oxygen Uptake in Rat Brain Slices or Kidney Slices

(For the experimental conditions, see Table I.)

Group	Brain cortex			Kidney cortex		
	n	Mean Q_{O_2}	Differ.*	n	Mean Q_{O_2}	Differ.*
Unoperated control	7	-8.3 ± 1.0**	—	7	-18.2 ± 3.1**	—
Epinephrinized	7	-7.4 ± 1.4	i.s.	7	-19.7 ± 2.3	i.s.
Anesth. with TCE	7	-6.8 ± 1.3	s.	7	-17.3 ± 1.4	i.s.
Anesth. with ether	5	-6.6 ± 1.2	s.	8	-17.4 ± 2.1	i.s.
CCl_4 administered	6	-6.9 ± 1.2	s.	6	-19.8 ± 1.3	i.s.

* This colum shows whether the mean differences between control cases and each experimental ones are significant (s.) or insignificant (i.s.) at the 5 per cent level of F-test.

** Confidence limit of the mean ($\alpha=0.05$)

on this problem are necessary. Oxygen uptake in kidney cortex did not practically differ by groups. It seems that the four substances examined do not exert any effects upon kidney cortex. In case of E. anesthesia the result agrees with that of previous worker (*8*).

TABLE III

Oxygen Uptake in Rat Liver Slices

(For the experimental conditions, see Table I)

Group	With butyrate			Without butyrate		
	n	Mean Q_{O_2}	Differ.*	n	Mean Q_{O_2}	Differ.*
Unoperated control	7	-12.4 ± 1.8**	—	6	-9.2 ± 0.9	—
Epinephrinized	6	-12.1 ± 1.3	i.s.	7	-9.2 ± 1.9	i.s.
Anesth. with TCE	7	-11.0 ± 1.4	i.s.	7	-8.4 ± 1.0	i.s.
Anesth. with ether	8	-9.9 ± 1.4	s.	8	-8.4 ± 1.1	i.s.
CCl_4 administered	7	-7.3 ± 2.3	s.	7	-5.6 ± 1.8	s.

*, ** See the foot-notes to Table II.

O_2-uptake in Liver—As it is shown in the table, average oxygen uptake in liver, with or without 0.003*M* butyrate, in control were -12.4 or -9.2, which are compared with Edson's average value of -14.1 or -10.9 (*23*), and -12.74 or -9.77 by Jowett and Quastel (*24*)

obtained by $0.01M$ butyrate under similar conditions. In case of CCl_4 administration group, there are a significant decrease of oxygen uptake both with and without butyrate, while in case of E. anesthesia group, the former decrease is exclusively significant. From these results it is conceivable that the oxygen uptake in liver is influenced greatly by CCl_4 administration, by E anesthesia it is influenced less than CCl_4, and that TCE and EP do not exert any effects upon it.

As for TCE, the result may correspond to Taylor's results that does not exert any histologically considerable influence on the liver and kidney of rat, exposed for a long time to TCE vapour (*25*).

II. Metabolism of Ketone Bodies in Liver Slices

Butyric Acid Consumed and Ketone Bodies Produced in Liver Slices—Amount of butyric acid consumed and amount of ketone bodies produced in liver slices were determined and compared by groups (Table IV).

TABLE IV

Butyrate Consumption and Ketone Bodies Production in Rat Liver Slices
(For the experimental conditions, see Table I)

Group	Butyrate consumed			Ketone bodies produced					
				With butyrate			Without butyrate		
	n	Mean†	Differ.*	n	Mean†	Differ.*	n	Mean†	Differ*
		μM			μM			μM	
Unoperated control...	7	35.2±3.8**	—	7	15.7±2.2**	—	7	6.9±1.6**	—
Epinephrinized.........	6	43.4±6.2	s.	7	19.2±3.1	s.	7	6.8±1.4	i.s.
Anesth.with TCE......	7	36.9±7.1	i.s.	7	18.2±3.9	i.s.	7	7.7±1.3	i.s.
Anesth. with ether ...	7	29.0±5.2	s.	7	12.6±2.2	s.	8	5.1±1.7	i.s.
CCl_4 administered ...	7	20.9±6.4	s.	7	7.7±3.3	s.	7	3.3±1.9	s.

† Represents values expressed in μM of butyrate consumed or total ketone bodies produced per 100 mg. of dry weight liver per 60 minutes.

*, ** See the foot-notes to Table II.

As shown in the Table, in control group the amount of butyrate consumed, and in that of total ketone bodies produced, with or without $0.003M$ butyrate, per 100 mg. of dry weight liver per 60 minutes, were 35.2 and 15.7 or 6.9 μM, respetively. The values of total ketone bodies were comparable with the averages of acetoacetate by Edson, 18.4 or 5.5 (*23*), and 20.3 or 4.3 by Jowett and Quastel (*24*), obtained by 0.01*M* butyrate. Both the amount of butyrate consumed, and of total ketone bodies produced with added butyrate, increase significantly in group of EP administration, and decrease significantly in cases of E anesthesia and CCl_4 administration. TCE did not exert any significant influence on them. A significant decrease of endogenous ketone bodies production was observed exclusively in group of CCl_4 administration.

General conclusion to be given from the results is that the production of ketone bodies in liver will be accelerated by EP administration, will be lowred by E. anesthesia or CCl_4 administration, and will not be influenced by TCE anesthesia. It is quite sure that ketosis observed by administration of EP (*26*) may be due to increased production of ketone bodies in liver.

Calculated O_2-uptake and Ketone Bodies Production—Amount of oxygen consumed to oxidize definite amount of butyrate, and amount of total ketone bodies produced from the latter, were calculated with the results summarized in Table V.

TABLE V

Calculated Oxygen Uptake and Ketone Bodies Production†

Group	Oxygen uptake			Ketone bodies produced		
	n	Mean	Differ.*	n	Mean	Differ.*
Unoperated control	6	0.37±0.10**	—	7	0.25±0.07**	—
Epinephrinized	7	0.37±0.12	i.s.	7	0.31±0.10	i.s.
Anesth. with TCE	7	0.37±0.07	i.s.	6	0.32±0.02	s.
Anesth. with ether	6	0.28±0.13	i.s.	7	0.25±0.03	i.s.
CCl_4 administered	7	0.36±0.19	i.s.	7	0.22±0.10	i.s.

† Represents extra oxygen uptake or ketone bodies production in μM divided by the amount of butyrate consumed in μM.

*, ** See the foot-notes to Table II.

These values will represent extra oxygen uptake or ketone bodies production (*i.e.*, increase caused by the addition of butyrate) divided by the amount of butyrate consumed. If the calculation conducted is valid, it is stated that in control cases one unit of butyrate takes up 0.37 units of oxygen molecule and produces 0.25 units of ketone bodies. The latter figure is compared with Jowett and Quastel's results of 0.22–0.26 units obtained in guinea-pigs (*24*). The ratio of the two values (extra ketone bodies production/extra respiration) is 0.68, as compared with Jowett and Quastel's average value of 0.63 obtained in guinea-pigs with $0.01M$ butyrate (*13*). As for oxygen uptake, there are no significant differences among five groups. In group of E anesthesia this value was 0.28, but the probability of difference against control group did not extend as far as the level of 5 per cent of F-test. While in groups of EP administration and TCE anesthesia, amounts of ketone bodies produced were 0.31 and 0.32, respectively. However, the increase was statistically significant in the EP group only. These results may indicate that the difference among groups in oxygen uptake or in ketone body production, as shown in Tables III and IV, respectively, except in case of TCE anesthesia, should be attributed rather to the quantitative different metabolic modes among groups. There remains, however, some doubt in the way of calculating the value due to the substrate from the difference between the values in presence and absence of substrate (*27*).

On the other hand Alexander referred already to the qualitative changes in gas metabolism due to E. anesthesia (*1*), besides it has been known that adrenal cortical hormone exerts qualitative change on metabolism of ketone bodies in liver slices (*16*). On this problem, further investigations are necessary.

III. Variations of Liver or Kidney Carbohydrate, and Liver Fat

Parts of orginal organs were taken out and the amounts of total and free carbohydrates in liver and kidney, and total fat in liver were measured. These results were summarized and compared by groups on Tables VI and VII.

Variations of Liver Carbohydrates—As will be seen from Table VI, figures for total and free carbohydrates in liver in control group were 1.77 per cent and 0.81 per cent, respectively, as compared with Yamamoto's result of 1.22 per cent and 0.86 per cent, obtained by rabbits fasted for 4 days (*21*).

These two values significantly decreased simulatneously by TCE or E anesthesia or by CCl_4 administration. In group of CCl_4 administration, these two values got their lowest level, and the matters should be attributed to the direct toxic action of CCl_4 on liver tissues. In groups of TCE or E anesthesia, decreases may be attributed, as reported by Evans and Brewster, partly to anoxia, to the direct action of

TABLE VI

Carbohydrate and Fat in Rat Liver

Group	Total carbohydrate			Free carbohydrate			Total fat		
	n	Mean	Differ.*	n	Mean	Differ.*	n	Mean	Differ*
		%			%			%	
Unoperated control...	7	1.77±0.32**	—	7	0.81±0.26**	—	7	4.3±1.2**	—
Epinephrinized.........	6	1.95±0.41	i.s.	6	0.88±0.32	i.s.	6	4.6±1.4	i.s.
Anesth. with T.C.E.	7	1.41±0.14	s.	7	0.51±0.12	s.	6	3.9±1.3	i.s.
Anesth. with ether ...	8	1.37±0.17	s.	7	4.48±0.10	s.	8	3.9±0.7	i.s.
CCl_4 administered ...	7	1.13±0.21	s.	7	0.26±0.11	s.	7	10.1±1.7	s.

*, ** See the foot-notes to Table II.

the anesthetics, and partly to the liberation of epinephrine from the suprarenals (*28, 29*). In the epinephrinized group, however, both total and free carbohydrates did not differ significantly from those of control groups, and the results agree with the author's previous work (*26*). The results may related to the promoted glyconeogenesis through the administration of E (*30*).

TABLE VII

Total and Free Carbohydrate in Rat Kindey

Group	Total Carbohydrate			Free carbohydrate		
	n	Mean	Differ.*	n	Mean	Differ.*
		%			%	
Unoperated control...............	7	1.35±0.11**	—	7	0.36±0.15**	—
Epinephrinized......................	7	1.39±0.16	i.s.	7	0.35±0.10	i.s.
Anesth. with TCE	7	1.34±0.16	i.s.	7	0.35±0.08	i.s.
Anesth. with ether	8	1.30±0.14	i.s.	7	0.29±0.05	i.s.
CCl_4 administered	7	1.22±0.14	i.s.	7	0.34±0.04	i.s.

*, ** See the foot-notes to Table II.

Variations of Kidney Carbohydrates—It is to be noted from the figures of Table VII that there is no significant difference between the groups in the amount of total and free carbohydrates in kidney.

Variations in Liver Fat—As indicated in Table VI, total fat in liver increased eminently in group of CCl_4 administration. However, among four other groups no significant differences were observed. In case of

E anesthesia this result may gree with that of Selbach, who reported of only slight fatty degeneration in the liver of dog or cat exposed to E anesthesia during 6–9 hours. (*31*).

According to Wymer (*2*), acidosis during anesthesia may be divided into two stages: primary or carbohydrate acidosis, and secondary or fat acidosis. The latter is caused by the disturbed oxidation of fat when anesthesia arrives at deeper stage which leads up to the shortage of glycogen. Taking into consideration of the high concentration of anesthetics employed in this experiment, and the low concentration of total carbohydrate in liver, these series of experiments on E anesthesia may be carried out beyond its stage of the secondary acidosis resulting to reduced metabolism of ketone bodies.

SUMMARY

Effects of two anesthetics, ether and trichloroethylene, on production of ketone bodies in slices of rats liver were investigated. Rats, fasted for 24 hours, and anesthetized, were autopsied and their tissue slices were prepared from liver, brain and kidney cortex. These slices were shaken as customarily in the Warburg apparatus for 60 minutes, and amount of oxygen uptake, and amount of ketone bodies produced or butyrate consumed in liver were determined. Meanwhile total fat in the original tissues of liver, and total and free carbohydrates in liver and kidney cortex were measured. Results were compared with those of the control, of the epinephrine and of CCl_4 administration group. Conclusions were given as follows:

1. Oxygen uptake in brain cortex is lowered by ether and trichloroethylene anesthesia, as by CCl_4 administration.

2. While both free and total carbohydrates of liver decrease in case of ether and trichloroethylene anesthesia, as in case of CCl_4 administration, those in kidney cortex are not influenced by the four substances administered.

3. Production of ketone bodies in liver is promoted by epinephrine administration, while it is reduced by ether anesthesia and CCl_4 administration, and is not influenced by trichloroethylene anesthesia.

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REFERENCES

(*1*) Alexander, F. G., and Cserma, S., *Biochem. Z.*, **53**, 100 (1913)

(*2*) Wymer, I., *Arch. klin. Chir.*, **143**, 200 (1926) ; *Deut. Z. Chir.*, **195**, 353 (1926)

(*3*) Nagai, R., *Masui* (*Japan. J. Anesth.*) **3**, 52 (1954) (in Japanese)

(*4*) Kohn, H. I., *Am. J. Physiol.*, **160**, 277 (1950)

(*5*) Reimann, S. P., and Bloom, G. H., *J. Biol. Chem.*, **36**, 211 (1918)

(*6*) Short, J. J., *J. Biol. Chem.*, **41**, 503 (1920)

(*7*) Jowett, M., *J. Physiol.*, **92**, 322 (1938)

(*8*) Enomoto, T., *Masui* (*Japan. J. Anesth.*) **3**, 109 (1954) (in Japanese)

(*9*) Jackson, D. E. *Anesthesia and Analgesia*, **13**, 198 (1934)

(*10*) Hewer, C. L., *Canad. Med. Assoc. J.*, **62**, 324 (1950)

(*11*) Saito, S., *Masui* (*Japan. J. Anesth.*) **2**, 72 (1953) (in Japanese)

(*12*) Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and tissue metabolism, Minneapolis, 2nd ed., 119 (1949)

(*13*) Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2143 (1935)

(*14*) Quastel, J. H., and Wheatley, A. H. M., *Proc. Roy. Soc.* (*London*) *B*, **112**, 60 (1933); Quastel, J. H., *Physiol. Rev.*, **19**, 135 (1939)

(*15*) Sugayama, S., oral report at the 74th annual meeting of the Japanese Pharmaceutical Society, Kyoto, April 1954

(*16*) Lipsett, M. N., and Moore, F. J., *J. Biol. Chem.*, **197**, 303 (1952)

(*17*) Greenberg, L. E., and Lester, C., *J. Biol. Chem.*, **154**, 177 (1944)

(*18*) Klink, L., *Biochem. Z.*, **273**, 1 (1934)

(*19*) Pozzi-Escot, M. E., *Bull., soc. chim. France*, [3], **31**, 932 (1904) ; cited from *Chem. Zentr.*, Bd II., s. 489 (1904)

(*20*) Bragdon, G. H., *J. Biol. Chem.*, **190**, 513 (1951)

(*21*) Yamamoto, C., *J. Biochem.*, **32**, 389 (1940)

(*22*) Bornstein, A., and Völker, H., *Biochem. Z.*, **209**, 103 (1929)

(*23*) Edson, N. L., *Biochem. J.*, **29**, 2082 (1935)

(*24*) Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2159 (1935)

(*25*) Taylor, H., *J. Ind. Hyg. Toxicol.*, **18**, 175 (1936)

(*26*) Niwa, M., *Kitasato Arch. Exp. Med.*, **26**, 185 (1953)

(*27*) Blumenthal, H., *Science*, **116**, 475 (1952)

(*28*) Evans, C. L., Tsai, C., and Young, F. G., *J. Physiol.*, **73**, 67 (1931)

(*29*) Brewster, W. R., Bunker, J. P., and Beecher, H. K., Am. *J. Physiol.*, **171**, 37 (1952)

(*30*) Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **79**, 309 (1928)

(*31*) Selbach, W., *Arch. exp. Pathol. Pharmakol.*, **34**, 1 (1894)

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STUDIES ON THE ACTION-PATTERN OF ANTI-TUBERCULAR SUBSTANCES

I. ANTIBIOTICS OF RHODOTORULA*

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Previously Okunuki (*1*, *2*,) reported that the oily product of *Rhodotorula Suganii* inhibits the growth of filamentous fungi. This antifungal substance (AF) was also found to inhibit the *in vitro* growth of *Mycobacterium tuberculosis humanus* as shown in Fig. 1. Chemical studies were carried out on this oily antibiotics and the substance was found to be oleic acid.

When *Rhodotorula Suganii* was incubated at 25° in a medium containing sugar and peptone, an antibiotic against *Staphylococcus aureus* was produced in 3 to 4 days, but its activity by the cup method was only 1 to 2 U./ml. compared with that of penicillin. The antibacterial action degraded after about a week, that was taken no notice of it. However, when the incubation was continued for two to three weeks, the production of the oily antibiotics became larger. Since the substance was produced inside the cells and soon exuded out, it could be extracted with some solvent from the cells and from the medium. When *Rh. Suganii* was incubated in liquid medium, the cells grew on the surface and proliferated, sinking to the bottom one after another. On such the stage, the yield of antifungal (AF) or antitubercular (ATB) substances was remarkable.

The yield of these substances in shaking culture was extremely low. The culture on agar plate produced a large amount of ATB and this was used for collecting ATB for chemical investigation. Sucrose or glycerol was most suitable as the carbon source for ATB-production, but not glucose, and potato was good for successive inoculation. Although the cells grew well, in medium of carrot, ATB was scarcely produced. This strain got into physiological "run down" in producing the antibiotics, but this phenomenon could be recovered by transplanting the cells on a potato medium. When the cells were preserved in a dry state in a desiccator over calcium chloride, ATB was fairly stable, but the stability decreased as the purification went on.

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* This work is dedicated to the late Prof. Dr. Keita Shibata and was published in the Miscellaneous Reports of the Research Institute for Natural Resources, No. **17**, **18**, 118 (1950) (in Japanese).

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Although some species of *Rhodotorula* yielded ATB in large or small quantities, *Rh. Suganii* showed the largest production of ATB.

EXPERIMENTAL

Studies of Culture Conditions for the Production of Antibiotics

Test of Growth Inhibition of Mycobacterium tuberculosis humanus—Respectively, 2 mg., 1 mg., 0.5 mg. and 0.2 mg. of the dried powder of *Rh. Suganii* were added to 10 ml. of the medium for *Mycobact. tuberclosis humanus,* H2 strain of the Institute for Infections Diseases, Tokyo, which had been incubated for three weeks. As shown in Fig. 1, the

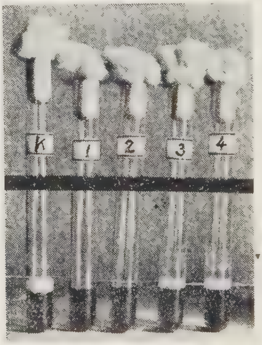


FIG. 1. Inhibition of the growth of *Mycobacterium tuberculosis humanus* by the dried cell-powder of *Rh. Suganii.*

The test tubes contained 0, 2, 1, 0.5, and 0.2 mg. respectively of the dried cell-powder of *Rh. Suganii* per 10 ml. of the culture-medium (glycerol broth) for *Mycobact. tuberculosis.*

K. control
1. 2 mg. of the powder 2. 1 mg. of the powder
3. 0.5 mg. of the powder 4. 0.2 mg. of the powder

growth of *Mycobact. tuberculosis humanus* was strongly inhibited at 1:10,000 dilution, even considering the evaporation of the medium.

Comparative Test of ATB-productivity of Several Species of Rhodotorula—Hereafter, for convenience, ATB-activity was judged by the maximum dilution, at which growth of *Mycobact. tuberculosis avium* was completely inhibited, being incubated at 37° during a week on the same medium for *Mycobact. tuberculosis* as described above. The medium for *Rh. Suganii* contained 2 per cent of sucrose, 1 per cent of peptone, 0.015 per cent of KH_2PO_4, 0.1 per cent of NH_4NO_3, 0.025 per cent of $MgSO_4$, and a trace of $FeCl_3$.

A flat-bottomed flask containing 200 ml. of the above medium, was sterilized, inoculated with each species of *Rhodotorula*, and incubated at 25° for three weeks. The ATB-productivity was compared using a) the dried powder of the grown cells, b) the ether-extract of the dried powder, and c) the ether-extract of the centrifuged broth. The grown cells were collected by centrifugation, placed on porous porcelain plate to remove the moisture, dired under vacuum in a desiccator, and the ground into a powder. The efficiency was tested with some portion of the powder and with the ether-extract of the powder by a Soxhlet extractor and dried under vacuum to remove the ether.

The centrifugate of the culture medium was tested with the ether-extract of the centrifugate, after complete removal of ether. The ether-extract was a red-colored semi-solid, some known weight of which was dissolved in a small amount of acetone, and a suitable volume of this acetone solution was added to the medium for *Mycobact. tuberculosis*, sterilized, and incubated with one drop of the suspension of *Mycobact. tuberculosis avium* as usual. The results are summarized in Table I.

Some strains other than *Rh. Suganii* yielded ATB and produced ether-extract containing little impurities, but only the strains of *Rh. Suganii* produced a large amount of ATB, the others a little. This result accords with that of AF-production reported previously (*1*).

Tests of Shaking Culture—Shaking culture was tried, substituting molasses for sugar in order to decide whether tank-culture is practicable or not. A mixutre of 200 ml. of the medium described above and 40 g. of molasses per liter was placed each of one-liter flask and shaking-cultured at 24–25° as usual. Each flask was subjected to efficiency-test after a definite interval of days, and the result is shown in Table II.

In shaking culture, only a small amount of the viscous substance was produced and the moisture was immediately absorbed when the cells were placed on a procelain plate. Therefore, this method is more convenient in treatment but much worse for ATB-production than the static culture. For tank culture, one should select some suitable strain which produces a considerable amount of ATB even in shaking culture, because both of the culture methods yielded almost the same amount of cells.

Test of Culture on Agar Medium—In a Roux flask, 100 ml. of the medium for *Rh. Suganii* and 2 per cent of agar were placed, sterilized in an autoclave, inoculated uniformly on the agar plate with 1 to 2 drops of the dense suspension of *Rh. Suganii*, and incubated horizontally during two to three weeks. About one gram of the cells per flask was collected. This method supplied ATB almost equal to that in the static

TABLE I

Comparison of the Yield and the Inhibition Efficiencies of Antitubercular Substances Produced by Various Strains

Strain	Cells of red yeast				Medium	
	Dried cell-powder		Ether-extract		Ether-extract	
	Yield (*g./l.*)	Efficiency*	Yield (*mg./g.*)	Efficiency*	Yield (*mg./l.*)	Efficiency*
Rhodotorula Suganii	4.3	2000	265	70000	260	70000
„ „ *var. α*	4.9	2000	133	70000	63	20000
„ *miniate*	0.5	500	155	100000	23	50000
„ *decolans*	0.5	< 500	50	< 10000		
„ *koishikawensis*	1.5	500	84	100000	32	< 3000
„ „ *var. α*	1.0	< 500		< 10000		
* *Shibatana*	4.2	500	10	30000	34	30000
„ „ *var. α*	4.7	< 500	9	30000	44	< 3000
„ *rubra*	0.3	< 500	55	30000	6	10000
„ *sauguinea*	4.4	< 500	59	50000	12	10000
„ *rufula*	4.9	< 500	37	100000	26	8000
Torula utilis	1.2	< 500	12	< 10000		

* Maximum dilution inhibiting growth.

TABLE II

Comparison of Culture Methods

Culture method	Culture days	Color of dried cell powder	Cells of red yeast				Medium	
			Dried cell powder		Ether extract		Ether extract	
			Yield (*g./l.*)	Efficiency*	Yield (*mg./g.*)	Efficiency*	Yield (*mg./l.*)	Efficiency*
Shaking culture	7	Grayish purple	5.5	500	16	40000	65	10000
	14	Chocolate	6.0	< 300	26	20000	65	10000
	21	„	5.5	< 300	28	4000	70	8000
	28	Gray black	5.0	< 300	80	10000	400	< 2000
Static culture	21	Purple	5.1	2000	100	50000	202	70000

* Maximum dilution inhibiting growth.

culture, and was adopted for following studies of the lipids.

Studies on the Lipid of Rh. Suganii

Extraction of the Lipid—The dried cells of *Rh. Suganii* were ground with sand and extracted with ethanol for 10 to 12 hours in a Soxhlet extractor. The extract was purple-colored, and when the ethanol was evaporated under vacuum (in hydrogen stream), viscous and dark reddish crude lipid was obtained. The dark brown substance was insoluble in ether and was removed by the following treatment: The crude lipid was dried under vacuum over night, shaken with a small amount of ether, and filtered. About three volumes of absolute acetone was added to the ether solution, were cooled, and white-colored phospholipid and wax that precipitated out filtered.

The filtrate was evaporated to about one-half the original volume and cooled by which acetone-insoluble, white-colored lipid precipitated. After filtration of the precipitate and evaporation of the acetone *in vacuo* (in hydrogen stream), dark red, semi-solid and acetone-soluble fat were obtained (Table III). This fat melted into dark purple liquid at about 25°, the properties of which were as follow:

Saponification value	208.7	(material 0.4009 g.: N/2 ethanolic KOH 3.02 ml.)
Acid value	148.7	(material 0.4009 g.: N/2 ethanolic KOH 2.13 ml.)
Ester value	60.0	
Iodine value	90.86	(material 0.1203 g.: N/2 $Na_2S_2O_3$ 8.6 ml.)

Separation of the Acetone-soluble Fat—

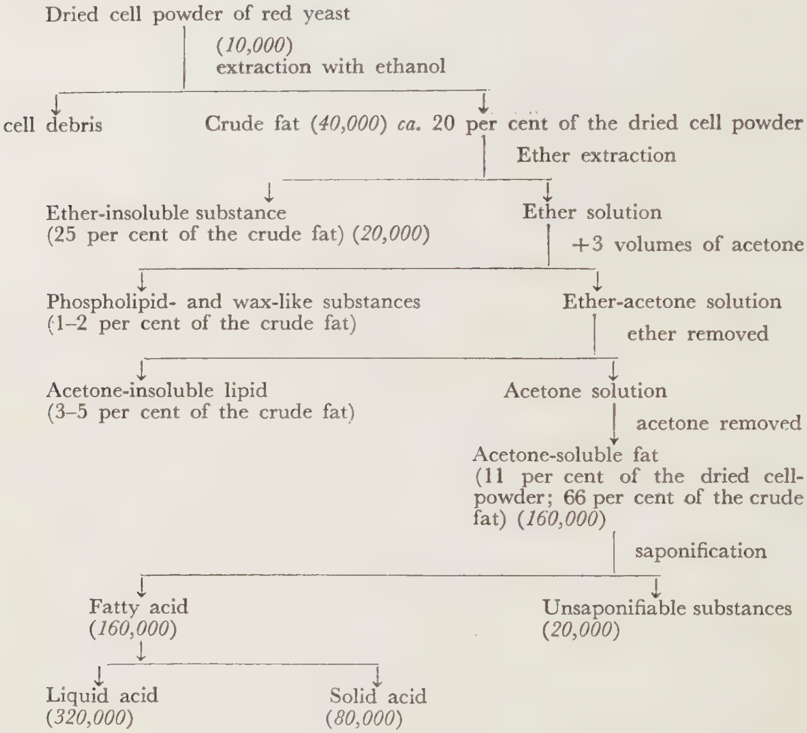
Saponification of the Fat: 18.3 g. of the acetone-soluble fat was dissolved in 50 ml. of 94 per cent ethanol, mixed with 160 ml. of *N*/2 ethanolic KOH, placed in a hydrogen stream for 30 minutes, boiled for a short time into a dark red solution, and ethanol was evaporated under a low pressure below 50°. By adding a great deal of ice-cold water, unsaponifiable matter was precipitated and was extracted with ether. The ether extract was washed with distilled water, evaporated, and the reddish orange unsaponifiable matter was removed by filtration. The filtrate was adjusted to weak acidity with 10 per cent HCl to precipitate fatty acids and the precipitate was extracted with ether. A total of 14.5 g. of crude fatty acid (76.6 per cent of fat) was obtained as an orange-colored semi-solid matter. The solution remaining after removal of the fatty acid was neutralized, concentrated to 1/10 the volume, and separated from inorganic substances by filtration. It was dried by evaporation *in vacuo* into a small amount of a crude substance soluble in water.

Separation of Fatty Acids: The fatty acid obtained by saponification had an iodine value, 83.7. This was separated into solid and liquid fatty acids by "lead salt-ethanol method." A solution of 14 g. of the crude fatty acid dissolved in 50 ml. of warm absolute ethanol was mixed with a solution of 2.05 g. of lead acetate in 80 ml. of absolute ethanol. The mixture was sealed closely with wax and preserved in an ice-box for a day in order to precipitate completely the lead salt of solid fatty acid, since white precititate appeared as the mixture chilled. The precipitate was filtered with suction, washed with absolute ethanol, and dried. When this lead salt of solid

TABLE III

Extraction of the Lipid

Italic numerals indicate the effective dilution inhibiting growth of *Mycobacterium tuberculosis avium.*)


Dried cell powder of red yeast
(10,000)
extraction with ethanol
cell debris
Crude fat (40,000) ca. 20 per cent of the dried cell powder
Ether extraction
Ether-insoluble substance
(25 per cent of the crude fat) (20,000)
Ether solution
+3 volumes of acetone
Phospholipid- and wax-like substances
(1–2 per cent of the crude fat)
Ether-acetone solution
ether removed
Acetone-insoluble lipid
(3–5 per cent of the crude fat)
Acetone solution
acetone removed
Acetone-soluble fat
(11 per cent of the dried cell-powder; 66 per cent of the crude fat) (160,000)
saponification
Fatty acid
(160,000)
Unsaponifiable substances
(20,000)
Liquid acid
(320,000)
Solid acid
(80,000)

fatty acid was decomposed by heating with HNO_3 and then extracted with ether, 4.2 g. of slightly yellow substance (29.9 per cent of fatty acid and 22.3 per cent of fat) was obtained. The filtrate of absolute ethanol was concentrated under a lower pressure in hydrogen stream below 60°, shaken with a large volume of 3 per cent HNO_3 to liberate liquid fatty acids, and extracted with ether. From the extract, 8.55 g. of a substance (61.1 per cent of fatty acid and 46.5 per cent of fat) was gained.

Solid Fatty Acids: By methylation of 4.58 g. of crude solid fatty acid (iodine value, 2.2) with diazomethane as usual, 4.6 g. of its methyl ester was obtained and fractionally distilled by the Klenk's apparatus :

		Yield
1st Fraction	b. p. 117–127°/0.02 mm. Hg	0.51 g.
2nd ,,	b. p. 127–139°/0.02 mm. Hg	1.81 g.
3rd ,,	b. p. 140–150°/0.02 mm. Hg	1.65 g.

By saponification of the first and the second fractions by boiling with 5 per cent ethanolic KOH for 5 minutes, 0.5 g. and 1.37 g. of free fatty acids were obtained, respectively. By recrystallization of both twice from methanol and once from acetone, crystals of m.p. 61° were gained. These crystals were confirmed as palmitic acid by admixture and by the following tests :

Sample : 7.722 mg.
0.01 N KOH required : 3.027 ml.
Calcd. for $C_{16}H_{32}O_2$: COOH, 17,56 ; mol. wt., 256.25.
Found COOH, 17,64 ; mol. wt., 255.06.

When the third fraction was saponified with 5 per cent ethanolic KOH in the same way, 1.56 g. of fatty acid was obtained whose recrystallization as above yielded scale-like crystals of m.p. 76∼77°. These were identified with arachidic acid by admixture and by the following tests :

Sample : 4.235 mg.
0.01 N KOH required : 1.333 ml.
Calcd. for $C_{20}H_{40}O_2$: COOH, 14.40 ; mol. wt., 312.3.
COOH, 14.17 ; mol. wt., 317.7.

Liquid Fatty Acid: By the usual method, the liquid fatty acid was derived to the methyl ester, a small amount of the ester was distilled *in vacuo*, and saponified into a substance, which had molecular weight and an iodine value approximately those of ocatdecenoic acid and a boiling poins similar to oleic acid, and was positive in elaidic acid-test as shown below :

Sample : 15.255 mg.
0.05 N $Na_2S_2O_3$ required : 2.199 ml.
Iodine value, 91.5
Sample : 14.060 mg. 0.01 N KOH required : 5.042 ml.
Calcd. for $C_{18}H_{34}O_2$ COOH, 16.07 ; mol. wt., 282.3
Found COOH, 16.12 ; mol. wt., 279.2

A solution of 0.2 g. of the methyl ester in ethyl acetate was reduced catalytically in the presence of platinum-black to a methyl ester, which was boiled with 5 per cent ethanolic KOH for 5 minutes and 0.16 g. of solid fatty acid, m.p. 66–67°, was obtained. The solid fatty acid was recrystallized from a mixture (3:1) of methanol and acetone to white scale-like crystals, m.p. 68–69°, which showed no depression in admixture with stearic acid.

Sample : 5.756 mg.

0.01 N KOH required : 1.666 ml.

Calcd. for $C_{18}H_{36}O_2$: COOH, 15.83 ; mol. wt., 284.3.

Found COOH, 15.77 ; mol. wt., 285.5.

Other fatty acids or saturated, liquid fatty acids were not found to exist in the reduction product. By the Klenk's apparatus, 4.62 g. of the methyl ester was fractionally distilled :

		Yield
1st Fraction	b. p. 133–135°/0.03 mm. Hg	0.45 g.
2nd ,,	b. p. 144–146°/0.03 mm. Hg	2.09 g.
3rd ,,	b. p. 153–158°/0.02 mm. Hg	0.92 g.

The first fraction was saponified with 5 per cent ethanolic KOH to liberate a liquid fatty acid having an value of 77.2, and a molecular weight of 312.9. *p*-Phenylphenathyl ester of this fatty acid of m.p. 59°, agreed with that of oleic acid. This fraction, therefore, must be oleic acid containing some impurities.

The second and the third fractions agreed with oleic acid in both the iodine value and molecular weight, and in the derivatives:

Sample : 10.650 mg.

0.05 N $Na_2S_2O_3$ required : 1.592 ml.

Iodine value, 90.2.

Sample : 11.017 mg.

0.01 N KOH required : 3.984 ml.

Calcd. for oleic acid : COOH, 16.07 ; mol. wt., 282.3.

Found COOH, 16.15 ; mol. wt., 277.9.

To a mixture of 300 mg. of this substance and 10 ml. of 4 per cent KOH was dropped in 8 ml. of 5 per cent $KMnO_4$ under cooling. After stirring the mixture for one hour, MnO_2 was filtered off and the filtrate was acidified with H_2SO_4. The precipitate thereby produced was recrystallized from 94 per cent ethanol and was identified by admixture with dihydroxystearic acid, m.p. 130°.

Estimation of Glycerol: The crude, water-soluble substance derived by saponification contained inorganic salts, and slightly brown, viscous substance was obtained from it by extraction with absolute methanol. This gave a positive ninhydrin reaction, gave acrolein-like odor on heating with conc. H_2SO_4, and positive to the reaction by KBr-salicylic acid (*3*) specific to glycerol.

Unsaponifiable Substance: 1.23 g. of the crude substance was dissolved in petroleum ether and allowed to stand in a cool place by which crystals separated which exhibited positive Liebermann's reaction (purple-violet-blue-green). The crystals were recrystallized from 94 per cent ethanol to white plates of m.p. 161.5° which showed no

TABLE IV

Antifungal Action of Oleic Acid extracted from Rh. Suganii

Concentration adden	Addition of oleic acid			Addition of the ether Extract		
	Weight of grown mycelia (*mg.*)	Average (*mg.*)	Ratio	Weight of grown mycelia (*mg.*)	Average (*mg.*)	Ratio
0	309 299 292	300	100	278 320 308	302	100
1 : 5,000	145 133 119 113	128	43	123 119	121	40
1 : 10,000	195 186 149 135	166	55	123 118	121	40
1 : 20,000	229 221 221 178	212	71	165 136	151	50
1 : 40,000	257 229 210 196	223	74	158 153	156	52
1 : 80,000	200 188 185 182	189	63	256 148	202	67
1 : 160,000	256 235 212 202	226	75	249 237	243	80
1 : 320,000	257 245 233 202	234	78	255 249	252	83
1 : 640,000	269 265 252 215	250	83			

depression on admixture with ergosterol.

The sterol-free, red petroleum ether solution was passed through an alumina column and the substance adsorbed was eluted out of each layer separately with methanol acidified with glacial acetic acid. Each of the eluates showed specific color reaction for carotenoids by using 85 per cent H_2SO_4, $SbCl_3$, conc. HCl-phenol, and fuming HNO_3. The fraction obtained from the chromatograph showed the color reaction for vitamin A by treatment with chloral hydrate (*4*).

AF-action of the Oleic Acid Derived from Rh. Suganii—A solution of the known weight of oleic acid and a small amount of acetone was added to the aforementioned medium for *Aspergillus oryzae*, sterillized, inoculated with some constant volume of the suspension of the conidia of filamentous fungi, and incubated for 96 hours at 25°. The dried cells were weighed, as shown in Table IV. This proved clearly that the oleic acid has AF-action. On the other hand, chemically synthesized oleic acid* was equal in the point of AF-action to oleic acid derived microbiologically (*5*).

ATB-action of the Lipid of Rh. Suganii—Table III shows the ATB-action of each fraction of the lipid of *Rh. Suganii* derived as described above. Arabic numerals express the maximum dilution inhibiting the growth of *Mycobact. tuberculosis avium.*

ACKNOWLEDGEMENT

We wish to express our deep appreciation to late Prof. Dr. Keita Shibata who assumed the leadership of this investigation. We thank the Ministry of Education for the Grant in Aid for Experimental Scientific Research which was delivered through the Japan Antibiotics Research Association to cover some of the cost for this research.

SUMMARY

An investigation was carried out on the antifungal and antitubercular activities of red yeast (*Rhodotorula Suganii*) and it was shown that the antimicrobial substances were contained in the acetone-soluble fat portion. The acetone-soluble portion was found to contain palmitic acid and arachidic acid as saturated fatty acids, and oleic acid as the unsaturated acid. Besides these acids ergosterol and cartenoids were also found to exist. Further chemical examination indicated that oleic acid, the main component of the active fat fraction, exhibits a remarkable antimicrobial activity *in vitro*.

REFERENCES

(*1*) Okunuki, K., *Japan. J. Botan.*, **5**, 402 (1931)

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* We wish to thank Prof. T. Isemura of the Institute of Scientific and Industral Research of Osaka Univ. for his kind supply of oleic acid.

(2) Okunuki, K., *Botan Mag. Tokyo*, **48**, 443 (1934) ; see Waksman, S. A., Microbial antagonisms and antibiotic substances, The Common Wealth Fund, New York, p. 185 (1945)
(3) Denigés, G., *Compt. rend.* **148**, 580 (1904)
(4) Livine, V. E., and Bien, G. E., *Proc. Soc. Exptl. Biol. Med.*, **32**, 873 (1935)
(5) Iwasa, K., and Okunuki, K., *J. Biochem.*, **41**, 695 (1954)

The Journal of Biochemistry, Vol. 41, No. 6, 1954

STUDIES ON THE ACTION-PATTERN OF ANTITUBERCULAR SUBSTANCES

II. ON THE FUNGISTATIC ACTION OF OLEIC ACID FROM RHODOTORULA

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In 1931, Okunuki (*1*), reported that *Aspergillus oryzae* grows very poorly in a culture medium in which *Rhodotorula* has been grown and the inhibitory action for growth was attributed to a metabolic product of *Rhodotorula*. As it is extractable with ether or acetone, he suggested that the substance or substances might be a lipoid.

In previous papers, Okunuki *et al.* (1950, 1954) (*2*, *3*) also reported that the antifungal substance remarkably suppressed the growth of *Mycobacterium tuberculosis avium* in glycerol-broth culture, and was confirmed by chemical examination to be oleic acid.

Recently, it has been reported by several investigators that some of saturated or unsaturated long-chain fatty acids cause growth inhibition of *Streptobacillius miniliformis* (*4*) and *Bacillus subtilis* (*5*), antisporulation in bacteria (*6*), growth stimulation of lactic acid bacteria (*7*), and abnormal mat formation of penicillia (*8*), but their rôle in the mode of action was very little known.

The present investigation was intended to clarify the mechanisms of antifungal and antitubercular action of oleic acid. From the data described below, it seems reasonable to conclude that the cause for these inhibitions may be some physical or physicochemical action rather than biochemical or antimetabolic reaction.

MATERIAL AND METHOD

Yeast Oleic Acid—Rhodotorula Suganii was cultured in a medium containing 20 g. sucrose, 10 g. peptone, 0.15 g. KH_2PO_4, 1.1 g. NH_4NO_3, 0.25 g. $MgSO_4 \cdot 7H_2O$, trace of $FeCl_3$ and 1000 ml. of distilled water, and if necessary, pH was adjusted to 5.6, in a Fernbach flask at 27°. After three or four weeks the cultured medium was shaken and extracted with ether in a separatory funnel and then the ether layer was evaporated on a water bath at about 40°.

The dried material, semi-solid fatty acids, mostly of oleic acid, was dissolved in acetone. The acetone-soluble portion, which possessed high antifungal and antitubercular activity, is designated as the " yeast oleic acid " in this paper. Yeast oleic acid was dissolved in a small amount of acetone, added to distilled water form an emulsion, and used in the present experiments. Pure oleic acid and other fatty acids were kindly supplied by Dr. Isemura.

Conidia of Aspergillus oryzae—Conidia of *Asp. oryzae* laboratory strain were used as the test organism for antifungal action. It was cultured for about 3 days in a flat aluminum vessel on a synthetic medium containing 50 g. sugar, 10 g. NH_4NO_3, 5 g. KH_2PO_4, 2.5 g. $MgSO_47H_2O$, trace of $FeCl_3$ and 1000 ml. of distilled water. The harvested mats of *Asp. oryzae* were floated in water for several minutes and dried in the air. The dried mats were sifted on Nr. 220 mesh. Some dried mycelia were occasionally present among the conidia that filtered through the mesh, but they had almost no biological activities. Fresh conidia of *Asp. oryzae* do not germinate uniformly. Therefore, the dried conidia were stored before use in a desiccator at room temperature for several months after harvest.

Surface growth experiments were carried out after Tamiya's glass-bell culture method (*9*) with some modifications. Each 5 ml. of the synthetic medium, which contained yeast oleic acid in various concentrations, was poured into a cylindrical glass vessel of 2.0 cm. in diameter and 3.5 cm. in depth, and covered with a bell-like glassware.

After the streilization, conidia were inoculated using the following three methods :

1) Thick seeding; Heavy inoculation by scattering the conidia through the mesh and floating them on the surface of the medium. 2) Thin seeding : Light inoculation by scattering the conidia a glass chamber using a mild air stream. 3) Suspending : Heavy inoculation by pippetting of conidia suspension.

After innoculated media were incubated at 27–30° for 48–50 hours, mats of fungus were floated on water, and after removing water on a filter paper, dried at 100–110° over night. The dried mats were chilled in a desicator before weighing.

Submerged growth experiments were performed by the usual method of bubbling air through a cotton stopper at 30°.

Gasometric Experiments on Surface Growth Culture—Tamiya's respirometer (*9*) shaped like an injection syringe was employed. The mats of *Aspergillus* cultured in cylindrical glass vessels at 27° for 23–24 hours usually harvested was 6.5–7.0 mg. dry weight. The mats were placed in the convex part of Tamiya's respirometer, in which 3 ml. of the sterilized medium was previously placed. Then, 100 ml. of N_2 gas or a mixture of 80 per cent N_2 and 20 per cent O_2 was filled in the gas chamber of the respirometers. After the respirometer was incubated at 27° for 38 hours, the gas was analyzed by Petterson's gasometer (*9, 10*).

The calculation from the analytical data and technical terms followed the Tamiya's reports (*9, 11*), *i.e.* I_N indicates CO_2 volume (ml.) evolved by aerobic oxidation, Q_N is I_N value per dry weight (mg.) of mat, I_I is CO_2 volume (ml.) evolved by anaerobic oxidation, Q_I is I_I value per dry weight (mg.) of mat, I_{co_2} is CO_2 volume (ml.) evolved by oxidation, and R.Q. is a ratio of I_{CO_2} to I_{O_2}.

Measurement of the Surface Tension—It was measured by the ring method of du Nöuy (*11*) and expressed as a relative value against distilled water at 18°.

Determination of Glucose—Hanes' iodemetry was employed (*12*).

Respiration of Conidia of Asp. oryzae—It was measured by means of Warburg's manometer by the usual method.

EXPERIMENTAL AND RESULTS

The effect of yeast oleic acid on the germination of conidia was tested as follows. On the plate medium containing 2 per cent agar and buffered to pH 5.6 with Na phosphate (final concentration *M*/20), a piece of cellophane smeared with conidia of *Asp. oryzae* was placed and incubated at 30°. Germination was observed every half an hour by a microscope in a warm room, the temperature of which was regulated to 30°. The result is presented in Table I.

TABLE I

Effects of Yeast Oleic Acid on the Germination of Conidia of Aspergillus Oryzae

Material added	Grade of germination of condia observed at successive times after inoculation.					
	3.0 hrs.	3.5 hrs.	4.0 hrs.	4.5 hrs.	5.0 hrs.	5.5 hrs.
Glucose	—	+	++	+++	++++	+++
Glucose and oleic acid	±	+	++	+++	++++	++++
Oleic acid	—	±	+	++	+++	+++
None	—	—	±	±	+	++

Final concentration of glucose, 1/40 *M*. Final concentration of yeast oleic acid, 2×10^{-4}. g/ml. Buffered with Na_2HPO_4-NaH_2PO_4 (final concn. 1/16 *M*.). Germination grade are indicated as follows ; — no germination, ± hardly any germination; + 20–30% germination ; ++ 30–50% germination ; +++ 50–80% germination ; ++++ almost total germination of living conidia.

As can be seen from Table I, yeast oleic acid is a promoter and not an inhibitor of germination of conidia.

The effect of yeast oleic acid on the resipiration of the suspended conidia was also examined by the ordinary Warburg manometer at 30°. Conidia of *Asp. oryzae* stored in a desiccator were weighed by a chemical balance, kneaded, and diluted with distilled water. In the main chamber of the Warburg manometer vessel, 0.5 ml. of the conidia suspension (about 2-5 mg. in dry weight) and 0.5 ml. of the synthetic medium described above, minus the glucose, added, while 0.5 ml. of glucose solution containing proper oleic acid emulsion was placed in the side arm, and 0.2 ml. of 20 per cent KOH in the well, with a piece of paper.

Results obtained with various concentrations of yeast oleic acid and glucose are shown in Table II.

TABLE II

Effect of Yeast Oleic Acid and Glucose on the Respiration of Conidia of Aspergillus oryzae

Expt. No.	Yeast oleic acid				Glucose			O_2-uptake for 5 hours (*micromoles*)	Excess O_2-uptake on addition of yeast oleic acid (*micromoles*) (c)	Oxidation promoted by addition of yeast oleic acid, *i.e.* $\frac{(c)}{(a)+(b)}\times 100\%$
	Final concentration (*M*)	Amount per vessel (*mg.*)	Amount per vessel (*micromoles*)	Theoretical O_2-uptake in complete oxidation (*micromoles*) (a)	Final concentration (*M*)	Amount per vessel (*micromoles*)	Theoretical O_2-uptake in complete oxidation (*micromoles*) (b)			
1	1/5,000	0.2	0.71	18.1	1/100	10	60	16.4	9.8	12.6
	1/10,000	0.1	0.35	9.1				12.6	6.0	8.7
	1/20,000	0.05	0.18	4.5				10.2	3.6	5.6
	1/40,000	0.025	0.09	2.3				7.8	1.2	1.2
	Nil	Nil	Nil	Nil				6.6		
2	1/5,000	0.2	0.71	18.1	1/100	10	60	17.2	9.6	12.3
	Nil	Nil	Nil	Nil				7.6		
	1/5,000	0.2	0.71	18.1	1/1,000	1	6	9.3	3.9	16.2
	Nil	Nil	Nil	Nil				5.4		
	1/5,000	0.2	0.71	18.1	1/10,000	1.1	0.6	6.2	2.8	14.9
	Nil	Nil	Nil	Nil				3.4		
	1/5,000	0.2	0.71	18.1	Nil	Nil	Nil	5.2	3.2	12.2
	Nil	Nil	Nil	Nil				3.0		

Conidia 2 mg. per Warburg vessel., pH 5.6. Temperature at 30°.

It will be seen from these data that yeast oleic acid itself is oxidized and O_2-uptake of conidia with glucose as a substrate is remarkably increased by the addition of yeast oleic acid. On the other hand, the rate of glucose disappearance from the medium is not affected by the addition of yeast oleic acid as shown in Fig. 1.

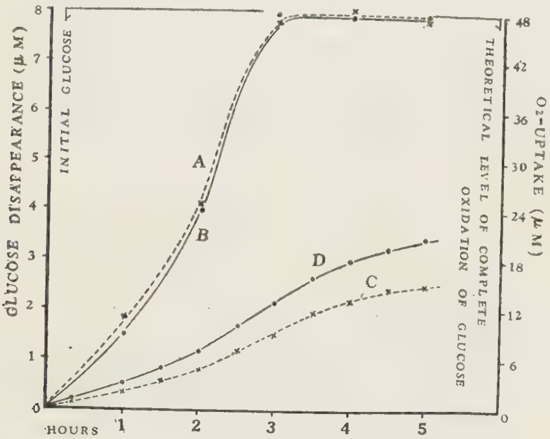

FIG. 1. Effects of yeast oleic acid on the consumption of glucose and the oxygen uptake by the conidia (*Aspergillus oryzae*).

Conidia : 5 mg. per Warburg vessel ; pH 5.6 ; Temperature, 30°. A and C, yeast oleic acid added (1 mg./ml. final) ; B and D, without yeast oleic acid. A and B show the glucose disappearance from the medium (8 micromoles per Warburg vessel in initial) ; C and D show O_2-uptake (endogeneous respiration have been deducted from both).

It appears that the oxidation of yeast oleic acid is accelerated by the addition of glucose (see Table II), as though the coupling oxidation between sugar and fatty acid *in vivo* may be a general metabolic phenomena. Though the actual O_2-uptake by conidia suspension was significantly higher in the presence of yeast oleic acid than that in its absence, it could account only for less than half of the amount of glucose that disappeared. From the interrelation between the disappearance and the oxidation of glucose, it seems reasonable to conclude that the glucose added might be not only oxidized by conidia of *Asp. oryzae* but also assimilated to some materials for utilization in germination.

The effect of yeast oleic acid on the experiment of surface growth was studied using the glass cylindrical vessels, and the results are shown in Figs. 2–7.

The culture inoculated with the suspension and the thin scattering revealed that the growth of mats was distinctly suppressed to the extent corresponding to the concentration of oleic acid, but in the culture with the heavy inoculation, no inhibition

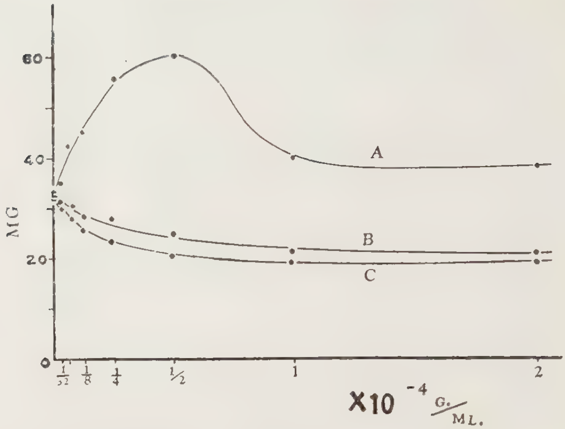

FIG. 2. Antifungal action of yeast oleic acid as it differs according to the inoculation method of conidia (*Aspergillus oryzae*).
Asbcissa, concentration of yeast oleic acid added to the synthetic medium ($\times 10^{-4}$ g./ml.). Ordinate, dry weight of two mats (mg.).
A : Thick scattering. B : Suspending. C : Thin scattering.

of mat growth was found (Figs. 2–5). Furthermore, in the case of the heavy inoculation on the medium by scattering, a low concentration of yeast oleic acid (1/20,000–1/160,000 g./ml.) promoted the formation of mat (Fig. 2). This phenomenon was not observed in the case of pure oleic acid (Fig. 3). The fact, therefore, suggests that yeast oleic acid is contaminated with a certain growth factor for *Asp. oryzae*.

It was always macroscopically observed that the mat grows like a ring only along the glass wall of the cylindrical vessel as a result of the fungistatic action (or antifungal action) of oleic acid in the case of surface culture.

The fact that the fungistatic action disappears by heavy inoculation suggest that the conidia of *Asp. oryzae* are physically immerged into the medium by yeast oleic acid and then the conidia cannot grow into a mat.

In order to confirm this hypothesis agar gel media were employed instead of a liquid one (Fig. 4).

The higher the agar concentration of the medium, the stronger was the disappearance of antifungal action. On the other hand, the surface tension of the medium containing various concentrations of yeast oleic acid was measured. An intimate correlation as shown in Fig. 5 was found between the lowering of the surface tension of the medium and the inhibition of the growth of mats.

The experiments with palmitic and myristic acids, which are long-chain saturated fatty acids, were performed under the same conditions. Contrary to the case of oleic

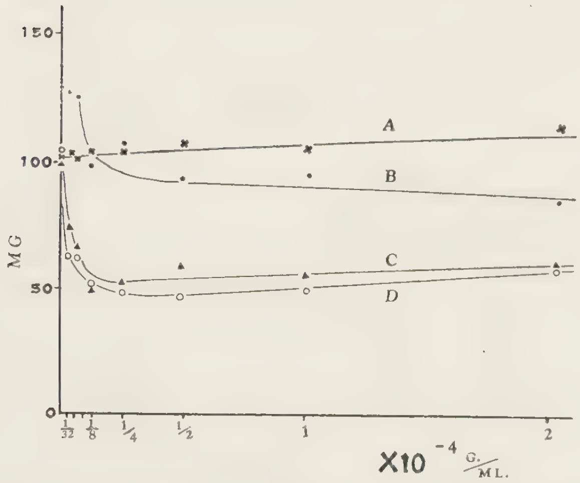

FIG. 3. Antifungal action of the pure oleic acid.

Abscissa, concentration of pure oleic acid added in the medium ($\times 10^{-4}$ g./ml.). Ordinate, dry weight of two mats (mg.).

A. Thin scattering, 0.4% agar added.
B. Thick scattering, no agar added.
C. Thin scattering, no agar added.
D. Suspending, no agar added.

acid, which is an unsaturated acid, neither antifungal activity nor lowering of the surface tension of the medium could be observed.

Conidia of *Asp. oryzae* were suspended for several minutes in acetone, ether, or ether containing yeast oleic acid, filtered through a paper filter, and the conidia were dried and inoculated by the thin scattered method. The experimental data are shown in Fig. 6.

The conidia washed with ether or acetone form mat normaly, but the conidia washed with ether containing yeast oleic acid are inhibited in the mat formation more strongly than the control. However, conidia treated with ether or acetone shows the same behavior as nontreated one in the manometric respiration and in the germination on an agar plate.

The effects of the yeast oleic acid on the submerged growth of *Asp. oryzae* was investigated using a culture in a 50 ml. glass bottle, with constant, aeration of air at 30°.

After about 10 hours' incubation, the conidia began to germinate, forming pellets. The data, as whown in Fig. 7, indicate that the growth of the pellets were inhibited about 25 per cent by the yeast oleic acid.

The effect of yeast oleic acid on the gas metabolism of the surface growth was

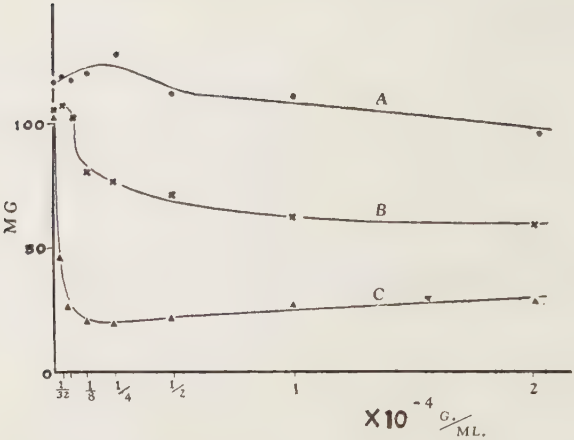

FIG. 4. Effect of the addition of agar on the antifungal action of oleic acid.

Abscissa, concentration of yeast oleic acid added in the medium ($\times 10^{-4}$ g./ml.). Oridnate, dry weight of two mats (mg.).

A. 1.2% agar added.

B. 0.6% agar added.

C. Control (no agar added). Inoculation method. Thin scattering.

tested by the Tamiya' respirometer and Petterson's gasometer. The results are shown in Fig. 8.

All the data given are mean values obtained in 4 to 6 experiments.

The data on the dry weight of the mats indicate that the younger the cells, the more distinct was the inhibition by yeast oleic acid. But there is no difference in Q_I or Q_N between the results obtained in the presence and absence.

When yeast oleic acid is added, the R.Q. values increase, especially distinctly in the initial stage of growth.

Antitubercular action of yeast oleic acid was also tested using *Mycobacterium tuberculosis avium* as the test organism. The bacteria cultured in glycerol broth (5 per cent glycerol) at 37° for seven days were collected from the medium, suspended in distilled water, and shaken in a flask with sterilized glass balls to form a milky suspension. A drop of the suspension was inoculated in glycerol-broth with and without agar.

Table III shows that in the medium containing 2 per cent agar the antitubercular action is not observed, even at the concentration of 2×10^{-4} g./ml., while the action appears at $1/4\times 10^{-4}$ g./ml. in the medium without agar (antitubercular activity=40,000).

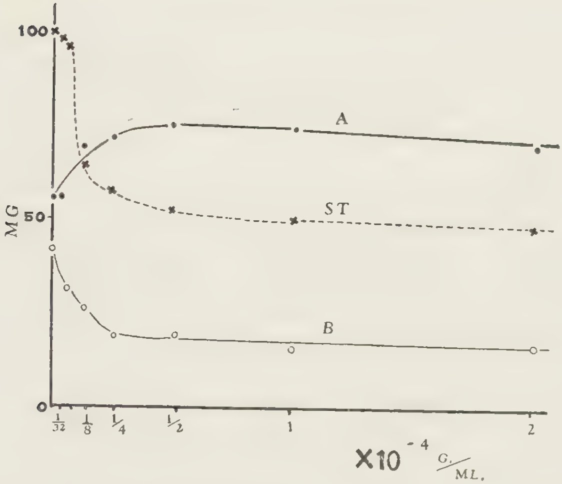

FIG. 5. Correlation between the antifungal action and the surface activity of yeast oleic acid.

Abscissa, concentration of yeast oleic acid added in synthetic medium ($\times 10^{-4}$ g./ml.). Ordinate, dry weight of two mats (mg.) and relative value of surface tension against distilled water at 18° (%).

A. Thick scattering of conidia.

B. Inoculation by suspension of conidia.

ST. Surface tension of the medium.

TABLE III

Effects of Agar on the Antitubercular Action of Yeast Oleic Acid

Yeast oleic acid *g./ml.*	2×10^{-4}	1×10^{-4}	$\frac{1}{2}\times 10^{-4}$	$1/4\times 10^{-4}$	$1/8\times 10^{-4}$	$1/16\times 10^{-4}$	$1/32\times 10^{-4}$	None
Glycerol-broth	−	−	−	−	+	+	+	+
Glycerol-broth plus agar (2.5% final concn.)	+	+	+	+	+	+	+	+

Glycerol-broth contains 0.25% meat extract, 1% peptone, 0.5% NaCl, 5% (w/v) glycerol ; pH 7.0. + indicates visible growth and —, non-detectable growth, in observation after four days' incubation at 37°. Assay organism, *Mycobacterium tuberculosis avium.*

DISCUSSION

By the present series of experiments it has been confirmed that the

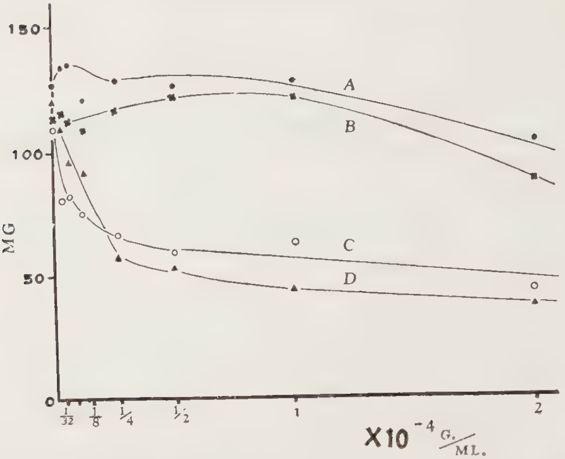

FIG. 6. Effect of pretreatment of conidia with acetone, ether, and ether containing yeast oleic acid.

Abscissa, concentration of yeast oleic acid added in the medium ($\times 10^{-4}$ g./ml.). Ordinate, dry weight of two mats (mg.).

A. Treatment with acetone.

B. Treatment with ether.

C. Treatment with ether containing yeast oleic acid.

Inoculation method. Thin scattering.

nature of the antifungal and antitubercular substances extracted from *Rhodotorula Suganii* agrees with that of oleic acid, because the pure oleic acid possesses the same actions as that of yeast oleic acid. It seemed that the antimicrobial action of yeast oleic acid could be attributed to a mechanism involving the change of metabolic pattern of the living organisms, in the same manner as that in which, 2,4-dinitrophenol or azide inhibits the oxidative assimilation, but the results of studies on the surface growth using agar plate and on the gas metabolism using Tamiya's respirometer and the Warburg manometer have denied such a possibility. Since oleic acid is an ordinary substance widely distributed in nature, it may not act as an antimetabolite or an enzyme inhibitor, but it may be assumed for several reasons that oleic acid might induce antimicrobial action secondarily by its physical or physicochemical properties. The available data supporting this assumption are as follows.

(1) The antifungal action or antitubercular action of yeast oleic

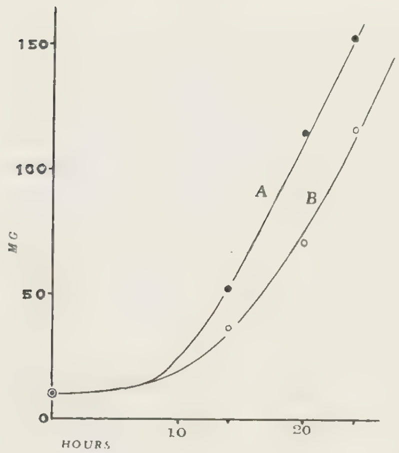

FIG. 7. Effect of yeast oleic acid on the growth of *Aspergillus* pellets in aeration culture.

Abscissa, time in hours. Ordinate, dry weight of pellets in mg.

A. Control (synthetic medium, *M*/5 glucose)

B. Yeast oleic acid added (yeast oleic acid, 2×10^{-4} g./ml. final.)

acid was seen only in a liquid medium, while the action was suppressed by the addition of agar, even at strongly inhibitory concentrations. On the basis of these data, it seemed reasonable to assume that the growth of the conidia of *Aspergillus* and the cells of *Mycobacterium* were inhibited by immersion into a medium.

(2) There is a fine correlation between the lowering of the surface tension of the medium and the growth inhibition in the mat formation if yeast oleic acid is added. In addition, it was ascertained that long chain fatty acids without the antifungal activity showed no lowering of the surface tension of the medium.

(3) By the effect of oleic acid the mat growth was especially markedly inhibited in the central portion of the surface of medium.

(4) The conidia treated with ether or acetone are not at all inhibited in the medium containing yeast oleic acid, while the one treated with ether containing yeast oleic acid is strongly inhibited.

On the other hand, it was observed that the R. Q. values in the surface culture with addition of yeast oleic acid were higher than that of the control. In view of the above fact, it appears that part of the

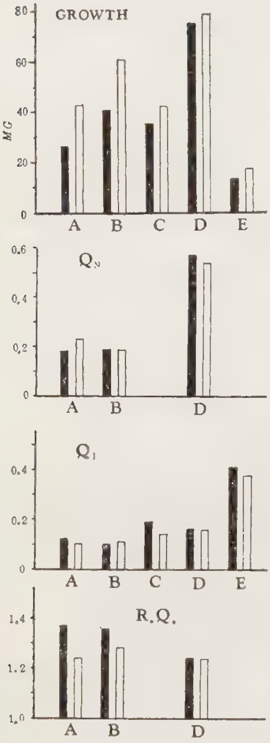

FIG. 8. Effect of yeast oleic acid on the growth and the gas metabolism of *Aspergillus oryzae* in surface culture.

Growth is expressed as dry weight of two mats (mg.).Q_N, CO_2 volume (ml.) evoluted by aerobic oxidation per dry weight (mg.), Q_I, CO_2 volume (ml.) evolved by anaerobic oxidation per dry weight (mg.). R.Q., respiratory quotient.

A. Mats formation from conidia for 48 hrs. at 30° under the aerobic condition.
B. Mats of A cultured for 8 hrs. at 30° undr the aerobic condition.
C. Mats of A cultured for 48 hrs. at 30° under the anaerobic condition.
D. Mats cultured in respirometer for 38 hrs. at 30° under the aerobic condition, after the conidia precultured to mats for 23 hrs. at 30° in the cylindrical vessels.
E. Same as D except for anaerobic condition.

Black bars show the addition of yeast oleic acid (1×10^{-4} g./ml.). White bars show the controls.

mats is wetted by the medium containing yeast oleic acid and kept in quasianaerobic condition.

It may be suggested from the results of these studies that the cells, conidia of *Aspergillus oryzae* and bacterial cells of *Mycobacterium tuberculosis avium*, are covered with lipid-like substances and, in a medium containing oleic acid, the cells are more easily wetted with the oleic acid emulsion by an affinity between the surface lipid of the cells and the lipophilic part of the oleic acid molecule. Hence it follows that the cells are more easily submerged into the medium and scarecely grow up.

Based on these considerations one might conclude that the antimicrobial action (antifungal and antitubercular action) of oleic acid could be attributed to a strictly aerobic property of the testorganisms.

SUMMARY

1. Mode of antifungal and antitubercular action of " yeast oleic

acid " was studied by several methods.

2. Oleic acid did not inhibit the germination of conidia and also the growth of tubercle bacilli on a plate medium.

3. The results of gas metabolism of conidia by the Warburg method was only found to be the coupling oxidation between glucose and oleic acid.

4. In the case of surface culture of the mat the presence of oleic acid did not affect Q_N and Q_I in the gas metabolism but increased RQ value to some extent.

5. In the experiment of surface culture, the inhibition of growth appeared only in a liquid medium, and the antimicrobial action disappeared by adding agar.

6. Formation of pellets of *Asp. oryzae* in aeration culture is inhibited by the addition of oleic acid.

7. From these results it was concluded that the antimicrobial action of oleic acid is due to its wetting action, by which the cells are submerged into the liquid medium, with result that the cells become incapable of respiring aerobically.

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REFERENCES

(*1*) Okunuki, K., *Japan. J. Botany*, **5**, 402 (1931) ; *Botan. Mag.* (*Tokyo*), **48**, 443 (1934)

(*2*) Okunuki, K., Matsukura, T., Shibata, S., Hiizumi, Y., Miura, Y., Toyoizumi, Y., and Kitamura, R., *Mis. Repts. Research Inst. Nat. Resources* (*Japan*), No. **17/18**, (1950).

(*3*) Okunuki, K., Matsukura, T., Shibata, S., Hiizumi, Y., Miura, Y., Toyoizumi, Y., and Kitamura, R., *J. Biochem.*, **41**, 683 (1954)

(*4*) Dumoff, M., and Duffy, C. E., *Proc. Soc. Exptl. Biol. Med.*, **77**, 1 (1951)

(*5*) Laser, H., *Biochem. J.*, **48**, 164 (1948), **51**, 57 (1952)

(*6*) Hardwick, W. A., Guikard, B., and Foster, J. W., *J. Bacteriol.*, **61**, 145 (1951)

(*7*) Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, **166**, 335 (1946); Williams, W. L., Broquist, H. P., and Snell, E. E., *J. Biol. Chem.*, **170**, 619 (1947); Broquist, H. P., and Snell, E. E., *J. Biol. Chem.*, **188**, 431 (1951)

(*8*) Ishida, Y., and Isono, M., *J. Antibiotics* (*Japan*), **5**, 327, 333, 377 (1952); Ishida, Y., Isono, M., and Suzuoki, Z., *J. Antibiotics* (*Japan*), **5**, 440 (1952); Ishida, Y., Isono, M., and Wakita, K., *J. Antibiotics* (*Japan*), **5**, 443 (1952)

(*9*) Tamiya, H., *Acta Phytochim.* (*Japan*), **4**, 227 (1928)

(*10*) Treadwell, F. P., Lehrbuch der analytischen Chemie, Bd. II, 11. Auf., Leipzig u. Wien, s. 329 (1930)

(*11*) Tamiya, H., *Acta Phytochim.* (*Japan*), **6**, 1, 227 (1932)

(*12*) du Noüy, *J. Gen. Physiol.*, **1**, 521 (1919)

(*13*) Hanes, C. S., *Biochem. J.*, **23**, 99 (1929)

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STUDIES ON THE ACTION-PATTERN OF ANTITUBERCULAR SUBSTANCES

III. OXIDATION AND OXIDATIVE ASSIMILATION OF GLYCEROL IN THE PRESENCE AND ABSENCE OF ANTIBIOTICS BY MYCOBACTERIUM TUBERCULOSIS AVIUM*

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It has been shown by a mumber of investigators that glycerol is very suitable as the carbon source for the culture of *Mycobacterium tuberculosis*. However, it seems most reasonable to conclude that glycerol is not an essential component of the culture medium, because Henley (*1*) and Dubos (*2, 3*) have used a culture medium containing no glycerol, and especially in *Mycobacterium tuberculosis avium* glucose can easily be substituted for it. On the other hand, Fernbach and Rullier (*4*), Henley (*1*), Merrill (*5*), and Corper, Cohn, and Bower (*6*) have reported that glycerol may be an indispensable metabolite for the bacteria. In either case, it is certain that glycerol plays a fairly important rôle in the growth of Mycobacteria. Although Sasagawa and Yamamura (*7*) have reported that glycerol is completely oxidized by the living cell-suspension of *Mycobacterium tuberculosis avium*, it seems reasonable to assume that glycerol can be oxidatively assimilated by the bacterium. In order to determine whether this assumption is tenable or not, the present investigation was undertaken to find out the mode of action of various inhibitors and antibiotics on the oxidation and the oxidative assimilation of glycerol, by comparing the metabolism of the living cells at various stages of culture.

EXPERIMENTAL

Methods and Materials

Bacterial Strain—Mycobacterium tuberculosis avium was used in the present series of investigation. The streptomycin-resistant cells were obtained by successive cultures of the sensitive strain.* The bacteria were incubated on glycerol-broth agar of pH 7.0, at 37° for 5 to 7 days. After that, according to usage, the grown cells were preserved at room temperature for some weeks, protecting the agar from drying.

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*This was read as a paper at the 23rd General Meeting of the Japanese Biochemical Society in Kobe, May, 1952.

Cell Suspension—The grown cells were harvested, centrifuged, ground in an agate mortar for 20 minutes, washed 5 times with distilled water, and finally suspended in distilled water to about 15 mg. of the dry weight per ml. The cell suspension could be kept in a refrigerator for a few days, but if the suspension was rewashed after the keeping, its ability to oxidize the added substrates decreased because some substances exuded out of the cells. The activity of the substance remained on boiling in water for 10 minutes and on dialysis against distilled water. Such a substance appeared to exude more when the cells were suspended in a physiological saline solution.

Enzyme Activities—These were measured using the Warburg manometric apparatus under the following conditions : The gas chamber contained air ; the vessel contained 1.0 ml. of the cell suspension, 0.4 ml. of $M/5$ phosphate buffer solution (pH 7.0) and others, the total volume adjusted to 2.2 ml. with distilled water ; water bath was regulated at 37°.

Estimations—Glycerol was determined photometrically by the method of Harvey and Higby (*8*). Carbohydrates were estimated photometrically with Dreywood's anthrone reagent (*9*). The substances measureable by this reagent were taken as the total carbohydrate.

Antibiotics—Streptomycin, Aureomycin, Terramycin, Chloromycetin, Flavomycin and Reticulin were used throughout.

RESULTS

Oxidation of Glycerol by the Cell Suspension—When 8 μM of glycerol were added to the cell suspension, about 9 μM of oxygen was consumed as shown in Fig. 1, whereas the complete oxidation requires 28 μM of oxygen. This result shows that only 1 mole of oxygen was takenup per mole of glycerol. Estimation revealed that glycerol remained in the reaction medium in (A) and (B) in the figure only in the amount of approximately 1/9 and less than 1/10, respectively, of the initial concentration. This fact indicates that glycerol has been transformed oxidatively into some other substance at an early stage of the oxidation.

Effects of Various Inhibitors on the Oxidation of Glycerol—Hydroxylamine

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* Strain of the Takeo Department for Tuberculosis, Institute for Microbial Diseases, Osaka University. The resistant substrain was kindly supplied by Dr. Hori of the Institute. The same were observed with the resistant substrain of the Institute and with the one which our laboratory attained.

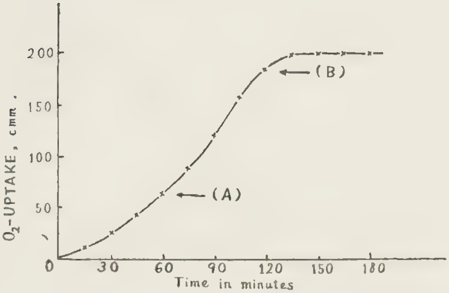

FIG. 1. Glycerol oxidation.

Each vessel contained 1.0 ml. of the suspension of streptomycin-sensitive cells which had been preserved at a room temperature for 2 days after 7 days' incubation, and 0.4 ml. of $M/5$ phosphate buffer solution (pH 7.0). Similar results were attained with streptomycin-resistant cells.

Solution of 8 μM of glycerol was added at the beginning and estimated at the points indicated by (A) and (B).

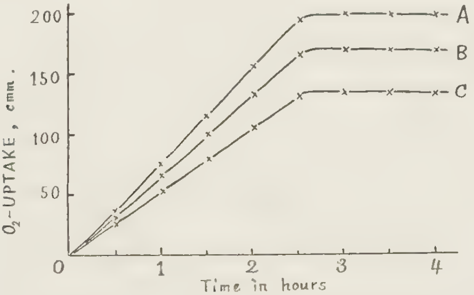

FIG. 2. Effects of hydroxylamine on glycerol oxidation with the streptomycin-sensitive cells suspended immediately after 5 days' incubation. Similar results were attained with streptomycin-resistant cells.

A. 8 μM of glycerol
B. 8 μM of glycerol and 1 μM of hydroxylamine
C. 8 μM of glycerol and 4 μM of hydroxylamine

inhibited the oxidation (Fig. 2), while 2,4-dinitrophenol accelerated it (Fig. 3). Malonate had no effect on it.

Effects of Various Antibiotics on the Oxidation of Glycerol—Streptomycin Reticulin, Chloromycetin, Aureomycin, Terramycin, and Flavomycin were used. All the antibiotics tested, except only Flavomycin, caused

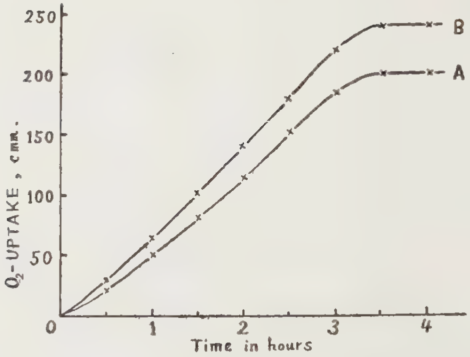

FIG. 3. Effects of 2,4-dinitrophenol on glycerol oxidation with the streptomycin-sensitive cells suspended immediately after 5 days' incubation. Similar results were obtained with streptomycin-resistant cells.

A. 8 μM of glycerol
B. 8 μM of glycerol and 0.2 μM. of 2,4-dinitrophenol

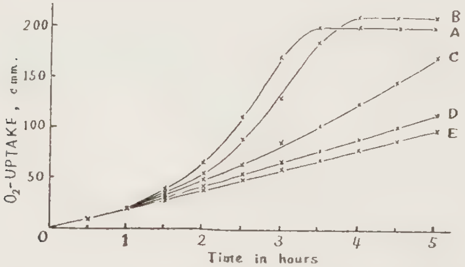

FIG. 4. Effects of Streptomycin on glycerol oxidation with the streptomycin-sensitive cells suspended after 7 days' incubation and 20 day's preservation.

A. 8 μM of glycerol
B. 8 μM of glycerol, and 0.1 γ/ml. of Streptomycin
C. 8 μM of glycerol, and 5 γ/ml. of Streptomycin
D. 8 μM of glycerol, and 20 γ/mt. of Streptomycin
E. 8 μM of glycerol, and 200 γ/ml. of Streptomycin

a delay of the oxidation-rate of glycerol. The effect of Streptomycin is shown in Fig. 4. However, when Streptomycin was added at a point maximum rate of the oxidation, the oxidation thereafter was not inhibited but rather slightly acclerated (Fig. 5). The oxidation-delay of glycerol was not observed with suspension of the cells which were resistant to Streptomycin of 1000 γ per ml. (Fig. 6).

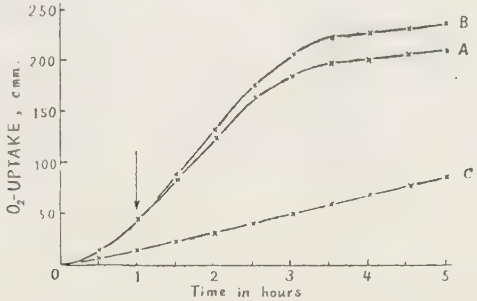

FIG. 5. Effects of the Streptomycin added at a point of maximum rate of glycerol oxidation.

With the streptomycin-sensitive cells suspended after 7 days' incubation and 10 days' preservation.

A. 8 μM of glycerol

B. 8 μM of glycerol, and 100 γ/ml. of Streptomycin added at the point indicated by an arrow.

C. 8 μM of glycerol, and 100 γ/ml. of Streptomycin added at the starting point.

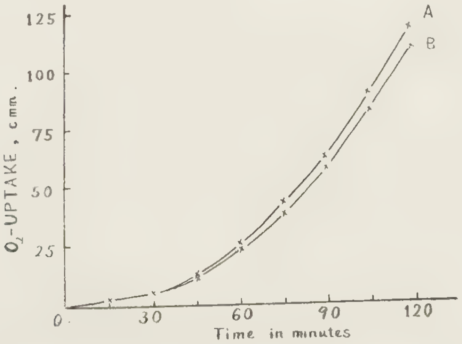

FIG. 6. Effect of Streptomycin on glycerol oxidation by the streptomycin-resistant cells. With the cells resistant against 1000 γ/ml. of Streptomycin, suspended after 7 days' culture and 10 days' preservation.

A. 8 μM of glycerol

B. 8 μM of glycerol, and 100 γ/ml. of Streptomycin preincubated for 30 minutes till the starting point.

Effects of Inhibitors and Streptomycin on Oxidative Assimilation of Glycerol —Quantitative analysis of carbohydrates suggested that glycerol and acetate were transformed into polysaccharides, the substances which were measurable with Dreywood's anthrone reagent (Fig. 7), and that the transformation of glycerol was much faster than that of acetate. Hydroxylamine, 2,4-dinitrophenol, and Streptomycin were found to inhibit its transformation. It seemed reasonable to conclude that glycerol and acetate might be oxidatively assimilated and the oxidative assimilation of glycerol might be inhibited by hydroxylamine, 2,4-dinitrophenol, and Streptomycin.

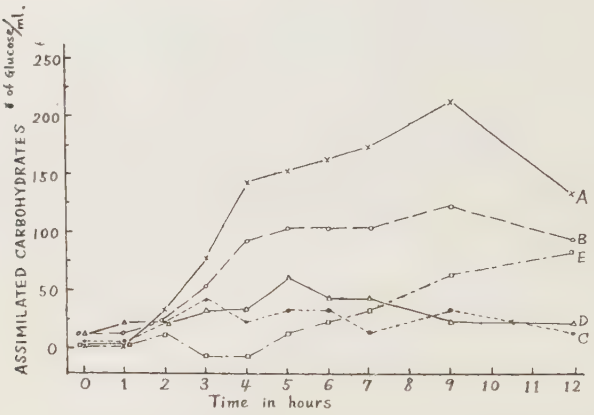

Fig. 7. Oxidative assimilation of glycerol and acetate, and the effect of 2,4-dinitrophenol, hydroxylamine, and Streptomycin on oxidation of glycerol. With the streptomycin-sensitive cells suspended after 5 days' incubation and 5 days' preservation ; the dry weight, 7.6 mg./ml. Each flask, capacity of 100 ml., contained 10 ml. of the cell suspension, 4 ml. of $M/5$ phosphate buffer solution (pH 7.0) and other solutions, the total volume adjusted to 20 ml. with distilled water. Shaken at 37°., 1 ml. of the content was separated every hour to estimate carbohydrates with Dreywood's anthrone reagent.

A. 40 μM of glycerol
B. 40 μM of glycerol and 4 μM of 2,4-dinitrophenol
C. 40 μM of glycerol and 4 μM of hydroxylamine
D. 40 μM of glycerol and 100 γ/ml. of Streptomycin
E. 40 μM of acetate

DISCUSSION

Sasagawa *et al.* (7) have reported that *Mycobacterium tuberculosis avium* oxidizes glycerol, which is important as the carbon source for its culture. However, it was not known whether glycerol is utilized only as an energy source or served in the synthesis of cellular matters. Our present results have demonstrated that a part of glycerol undergoes transformation into carbohydrates detectable by Dreywood's anthrone reagent, perhaps by oxidative assimilation. Although our results are not sufficient enough to regard glycerol as being essential for the growth of the bacteria, it is notable at least that glycerol was transformed into carbohydrates faster than acetate.

Glycerol was oxidized adaptively, showing a sigmoid curve. The longer the preservation at room temperature after incubation, the more adaptively occurred the oxidation of glycerol by the cells (compared with all of the figures). On the other hand, such phenomena could not be observed with a suspension of younger cells which were cultivated only for short time and were just proliferating at the harvesting time. The oxidation of glycerol was inhibited by Streptomycin and delayed in rate, and in the presence of a higher concentration of the antibiotic, it proceeds linearly at the same rate as at the start. Accordingly, inhibition of Streptomycin was demonstrated more clearly with the older than with the younger cells. With the streptomycin-resistant cells, effect of Streptomycin could not be demonstrated either with the younger or older cells, although the oxidation of glycerol by the older cells proceeded along a sigmoid curve. The foregoing results suggest that the enzyme system oxidizing glycerol may involve at least one enzyme which has both properties of the so-called constitutive and adaptive enzymes and which rises and falls in accordance with the state of the cells.

SUMMARY

1. *Mycobacterium tuberculosis avium* oxidized a part of the glycerol added and oxidatively transformed some part of the rest into carbohydrates such as saccharides, perhaps through oxidative assimilation.

2. The oxidation of glycerol by streptomycin-sensitive cells was accelerated by 2,4-dinitrophenol and by Streptomycin, and inhibited by hydroxylamine.

3. With the streptomycin-sensitive cells, Streptomycin affected to

delay more strictly the oxidation of glycerol by the older cells than by the younger cells, but such effect was not observed regardless of the ages with the streptomycin-resistant cells.

4. The transformation, perhaps oxidative assimilation, of glycerol of the sterptomycin-sensitive older cells was much faster than that of acetate.

The authors express their sincere thanks to Prof. S. Hosoya of the Institute for Infections Diseases, Tokyo University, Tokyo, to Prof. W. Aiso of the Institute for Putrefaction Research, Chiba, and to Prof. H. Umezawa of the National Institute of Health, Tokyo, who kindly supplied the pure antibiotics. The authors also express their thanks for the Grant in Aid for Experimental Scientific Research from the Ministry of Education, delivered through the Japan Antibiotics Research Association, to defray a part of the expenditure for the research of this series.

REFERENCES

(*1*) Henley, R. R., *Am. Rev. Tuberc.*, **32**, 724 (1935)
(*2*) Dubos, R. J., *Proc. Soc. Exptl. Biol. Med.*, **58**, 361 (1945)
(*3*) Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, **56**, 334 (1947)
(*4*) Fernbach, E., and Rullier, G., *Compt. rend. soc. biol.*, **131**, 1142 (1939)
(*5*) Merrill, M. H., *J. Bacteriol*, **21**, 361 (1931)
(*6*) Corper, H. J., Cohn, M. L., and Bower, C., *J. Lab. Clin. Med.*, **25**, 981 (1940)
(*7*) Sasagawa, T., and Yamamura, Y., *Med. J. Osaka Univ.* **1**, 6 (1949)
(*8*) Harvey, S. C., and Higby, V., *Arch. Biochem. Biophys.*, **30**, 14 (1951)
(*9*) Morris, D. L., *Science*, **107**, 254 (1948)

The Journal of Biochemistry, Vol. 41, No. 6, 1954

STUDIES ON THE ACTION-PATTERN OF ANTITUBERCULAR SUBSTANCES

IV. ON THE METABOLISM OF MALONIC ACID BY MYCOBACTERIUM TUBERCULOSIS AVIUM*

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(Received for publication, August 15, 1954)

Most of the enzymes taking part in the tricarboxylic acid (TCA)-cycle were extracted cell-free out of *Mycobacterium tuberculosis avium* by Yamamura *et al.* (*1*), who insisted that the TCA-cycle is operating in the microorganism. For instance, the cell-free extract of the microorganisms oxidized succinate and the oxidation was inhibited by the addition of malonate or of sodium azide. The addition of malonate should influence the oxidation of succinate by the cell-suspension of *Mycobacterium tuberculosis avium*. Nevertheless, when added together, malonate itself is oxidized by the cell-suspension and does not at all inhibit the oxidation of succinate. Looking at its chemical structure, it is rather natural that malonate is metabolizable. Hayaishi, *et al.* (*2*) reported that uracil is cleaved into malonic acid and urea by certain bacteria. It may be probable that malonic acid is flowing within microorganisms as an intermediate in decomposition of nucleic acids, and in fact, it is known that malonic acid is a competitive inhibitor against succinic dehydrogenase.

Therefore, living organism, especially at a resting state similar to spores, may be regulated by the mutual relation between malonic acid and succinic dehydrogenase. This report describes the metabolism of malonic acid by the suspension of the living cells of *Mycobacterium tuberculosis avium*.

EXPERIMENTAL

Material and Method—*Mycobacterium tuberculosis avium* was incubated on glycerol

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bouillon agar of pH 7.0, during 5 to 7 days at 37°. The cell suspension was prepared by the same method as described in our previous report (*3*).

Estimation of Acetate—It was identified as its hydroxamic acid by paper chromatography (*4*, *5*) developed with butanol.

Enzyme Activity was measured by the Warburg manometric apparatus.

RESULTS AND DISCUSSION

The cell suspension oxidized the malonate added as a sole substrate as shown in Fig. 1, in which CO_2-evolution went fairly ahead of O_2-uptake. Therefore, malonate appeared to be at first decarboxylated into acetate. The reacting medium after 2 hours in the figure contained a certain fatty acid, the hydroxamic acid of which was identified with that of acetic acid by paper chromatography. The oxidation and the decarboxylation of malonate proceeded along a sigmoid curve and addition of Streptomycin inhibited both of them, as shown in Fig. 2, but not those of acetate. Comparison between acetate and malonate in the points of O_2-uptake and CO_2-evolution is shown in Table I. The malonate consumed almost the same amount of oxygen as that of acetate and evolved just one mole of carbon dioxide per mole of sub-

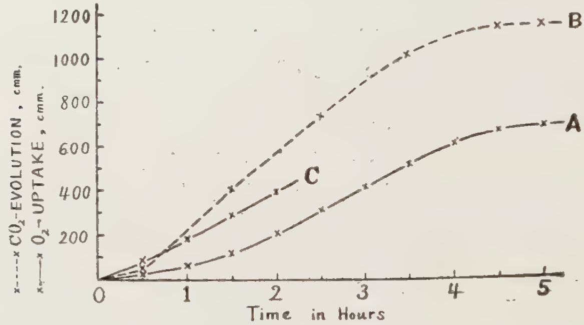

FIG. 1. Oxidation and decarboxylation of malonate by the cell suspension of *Mycobacterium tuberculosis avium.*

Each vessel contained 1.0 ml. of the cell suspension, 0.4 ml. of phosphate buffer solution (*M*/5, pH 7.0) and the substrate. Oxidation was measured by 0.2 ml. of 20% KOH in the center well and decarboxylation was done by 0.2 ml. of 6 moles of H_2SO_4 in the second side-arm, as usual. Temperature, 37°. A. 20 μM of malonate. B. 20 μM of malonate. C. 20 μM of acetate.

strate in excess of acetate. All of the malonate added to the cell suspension appeared to be transformed into acetate. Streptomycin would inhibit the adaptive formation of malonic decarboxylase which catalyzes decarboxylation of malonate into acetate.

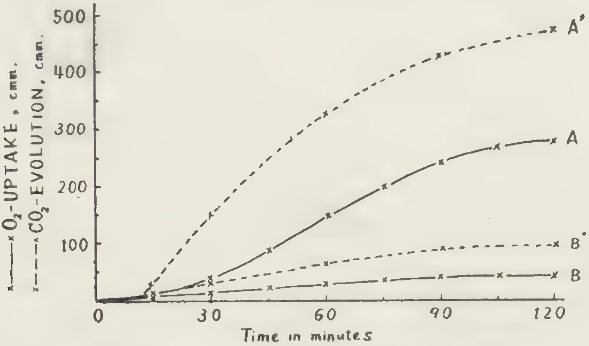

Fig. 2. Effect of Streptomycin on the oxidation and decarboxylation of malonate by the cell-suspension of *Mycobacterium tuberculosis avium*.

Experimental conditions were equal to Fig. 1.

A. 8 μM of malonate
A'. 8 μM of malonate
B. 8 μM of malonate + 500 γ/ml. of Streptomycin
B'. 8 μM of malonate + 500 γ/ml. of Streptomycin

TABLE I

Comparison between Malonate and Acetate Oxidation
(Concentration of substrate, 2×10^{-5} *M*.)

Substrate	Acetate	Malonate
O_2 consumed, *cmm.*	660*	650*
” ” , *moles/mole of substrate*	3/2	3/2
CO_2 evolved, *cmm.*	650*	1100*
” ” , *moles/mole of substrate*	3/2	5/2

* This is the value at which the reaction just arrived to a state of equilibrium.

From this point of view, the cell suspension adapted to malonate should produce carbon dioxide out of the malonate added under an-

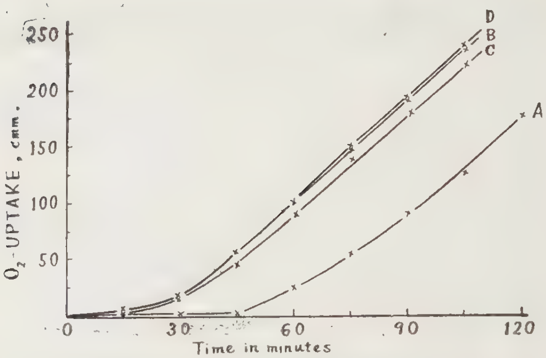

FIG. 3. Oxidation of malonate and acetate by the cell suspension previously preserved in N_2-atmosphere.

Both of the cell suspension adapted and not adapted to malonate were prepared in vessels as for Fig. 1 and the air inside the vessels was exchanged for N_2. After a half hour of shaking at 37°, the N_2 was rapidly exchanged again for air to measure the oxidation.

A and B, with the non-adapted cell-suspension

C and D, with the adapted cell-suspension

A. 8 μM of malonate

B. 8 μM of acetate

C. 8 μM of malonate

D. 8 μM of acetate

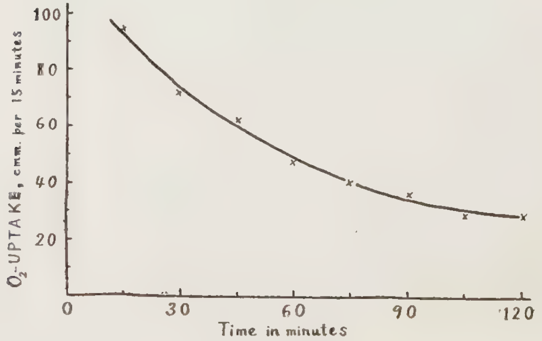

FIG. 4. Influences of preserving the cell-suspension in N_2-gas to its endogeneous respiration.

This result is a part of the experiment in Fig. 3. The curve was drawn along every 15 minutes of Xo_2.

aerobic condition. However, such CO_2-evolution could not be demonstrated in N_2, but the cell suspension which had been preserved in N_2 for a half hour oxidized malonate and acetate in air along strictly S-shaped curves as shown in Fig. 3, in spite of that acetate was oxidized constitutively by the cell suspension not preserved in N_2 (Fig. 1). In addition, the endogeneous respiration was much higher just after exchanging N_2 for air than prior to preserving in N_2, and restored to its original state, running down parallel to the restoration of the oxidations of acetate and of malonate, as shown in Fig. 4. These phenomena suggested that the cells present in N_2 are in some other state than that of the cells present in air, at which the cells do not decarboxylate malonate.

As described above, the oxidation and the decarboxylation of malonate were inhibited in the presence of Streptomycin. Such inhibition, however, could not be observed with a suspension of the streptomycin-resistant cells which grew well in the presence of 1000 γ of Streptomycin per ml. of the culture medium, as shown in Fig. 5.

Mycobacterium tuberculosis avium oxidizes benzoic acid into catechol by an adaptive enzyme, the formation of which is inhibited by Strepto-

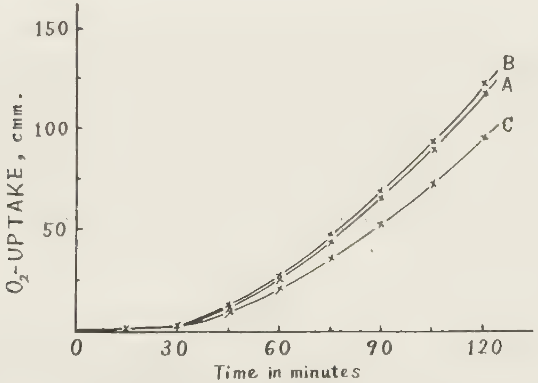

FIG. 5. Oxidation of malonate in the presence of streptomycin by the streptomycin-resistant cell-suspension.

Experimental conditions equal to those for Fig. 1 except that Streptomycin was added half an hour before the addition of the substrate.

A. 8 μM of malonate

B. 8 μM of malonate + 25 γ/ml. of Streptmycin

C. 8 μM of malonate + 500 γ/ml. of Streptomycin

mycin (*6*). In the streptomycin-resistant cells, however, its formation was not affected (Fig. 6). In the previous report (*3*), it was shown that the enzyme which oxidize glycerol important for the culture of *Mycobacterium tuberculosis* rises and falls according to the transition of cellular state; the younger cells are full of the enzyme, which degenerates as the cells grow older and the resynthesis of that in the older cells sensitive to Streptomycin is inhibited in the presence of Streptomycin, but not in the older cells resistant to Streptomycin. Therefore, it may be rational to consider two possibilities for the acquisition of "streptomycin-resistance": 1) rearrangement of metabolic pathway without streptomycin-sensitive enzymes and 2) formation of the enzymes even in the presence of Streptomycin.

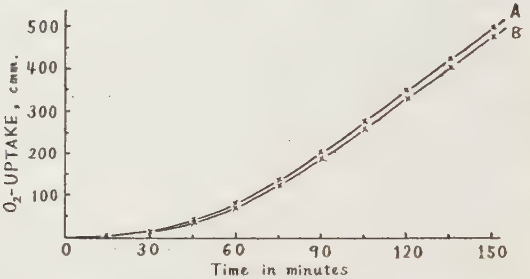

FIG. 6. Influence of Streptomycin to the oxidation of benzoate by the streptomycin-resistant cell-suspension.

Experimental conditions were the same as those for Fig. 5.

A. 8 μM of benzoate

B. 8 μM of benzoate+500 γ/ml. of Streptomycin

SUMMARY

1. The metablism of malonate was investigated using the cell suspension of *Mycobacterium tuberculosis avium.*

2. The cell suspension oxidizes malonate, which is at first step decarboxylated into acetate.

3. The enzyme taking part in the decarboxylation is an adaptive enzyme, the formation of which in the streptomycin-sensitive cells is inhibited in the presence of Streptomycin.

4. The cell suspension of the streptomycin-resistant substrain

oxidizes adaptively malonate and benzoate along S-shaped curves similar to the streptomycin-sensitive cells and these oxidations are not inhibited in the presence of Streptomycin.

REFERENCES

(*1*) Yamamura, Y., *Sogo Igaku*, **9**, 359 (1952) (in Japanese)
Yamamura, Y., Kusunose, M., and Kusunose, E., *J. Biochem.*, **39**, 227 (1952)
(*2*) Hayaishi, O., and Kornberg, A., *J. Biol. Chem.*, **197**, 717 (1952)
(*3*) Horio, T., and Okunuki, K., *J. Biochem.*, **41**, 709, (1954)
(*4*) Inoue, Y., and Noda, M., *J. Agr. Chem. Soc. Japan*, **24**, 291 (1951)
(*5*) Inoue, Y., and Noda, M., *J. Agr. Chem. Soc. Japan*, **24**, 295 (1951)
(*6*) Yamamura, Y., *Med. J. Osaka Univ.*, **1**, 1 (1949)

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STUDIES ON THE ACTION-PATTERN OF ANTITUBERCULAR SUBSTANCES

V. ON THE C_4-DICARBOXYLATE-METABOLISM OF MYCOBACTERIUM TUBERCULOSIS AVIUM*

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The mode of action of Streptomycin, as well as other antitubercular substances is yet obscure though it was found remarkably effective by clinical experiments. Numerous investigations have been carried out to find the possible sides of Streptomycin inhibition. It is natural to consider that Streptomycin have some influences at several points of living function, because it is a comparatively large and complex molecule.

In growth experiments, an extremely low concentration of Streptomycin is enough to prevent bacterial growth ; for example, 0.25 to 1 γ of Streptomycin per ml. of culture medium against *Mycobacterium tuberculosis avium.* Therefore, inhibitions caused by a higher concentration of Streptomycin must be beyond the object of pursuit in the investigation for the mode of action of Streptomycin against growth.

Growth inhibition of tubercule bacilli by substances follows two kinds of patterns as follows : (a) Growth is inhibited by the summation of various kinds of influences too weak to prevent growth by itself ; (b) One or two kinds of influences at some points in living function invite growth inhibition.

The present state of biochemistry is capable of advancing only on the assumption that growth inhibition is caused by (b).

Umbreit (*1*) studied the mode of action of Streptomycin with *Escherichia coli* from a similar stand-point, and reported that some reaction between pyruvate and oxalacetate is remarkably inhibited in the presence of a minimum concentration of Streptomycin to prevent the growth of the bacteria. In *Mycobacterium tuberculosis avium*, Yamamura *et al.* (*2*) have certified that all of the enzymes concerned with

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* This work was supported by the Governmental Grant for Experimental Scientific Research and reported in the meeting of the botanical Society of Japan at Kanazawa in October, 1953.

TCA-cycle are extractable in a cell-free state, and proposed a TCA-cycle functional in microorganisms. With *Mycobacterium tuberculosis avium*, we could not, however, demonstrate the oxalacetate-pyruvate reaction as Umbreit has done with *Escherichia coli*. On the contrary, it was found that the oxidation of C_4-dicarboxylates by *Mycobacterium tuberculosis avium* was strictly inhibited in the presence of a low concentration of Streptomycin.

EXPERIMENTAL

Bacterial Strain and Culture—*Mycobacterium tuberculosis avium* grown on the glycerol bouillon agar was utilized throughout this investigation. " Living Cell-Suspension " was prepared as described in the previous report (*3*).

The Younger Cells—The Younger cells are designated as 3 to 4 days' cultured cells, which are just proliferating, on log-phase, at harvest.

The Older Cells—Older cells are the 5 to 7 days' cultured cells preserved for more than half a month in a dark room, at a lag-phase of proliferation, under a condition to maintain the agar wet.

RESULTS AND DISCUSSIONS

The Oxalacetate-Pyruvate Reaction—Using the cell suspension, inhibition of Streptomycin against the oxalacetate-pyruvate reaction as Umbreit observed with *Escherichia coli* can not be demonstrated at all, while a simple summation of both of oxidation of oxalacetate plus Streptomycin and that of pyruvate plus Streptomycin is merely observed as shown in Fig. 1.

Acetate and malonate used instead of pyruvate give similar results.

Mycobacterium tuberculosis avium has a strong power of oxalacetate decarboxylation, which is extractable with $M/10$ phosphate buffer solution pH. 7.0 after grinding the cells with quartz powder. The cell-free extract catalyzes decarboxylation of oxalacetate in excess of its auto-decarboxylation in N_2-gas as shown in Fig. 2. Therefore, the oxalacetate will be decarboxylated into pyruvate immediately after addition to the living cell-suspension. In *Escherichia coli*, such an oxalacetate-decarboxylation can be prevented by preserving the cell suspension in a refrigerator and repeated washing, but that is practically difficult in *Mycobacterium tuberculosis avium*. However, the cell suspension which has been preserved for several days without washing oxidizes malate, and its oxidation has been confirmed to be somewhat inhibited in the presence of Streptomycin.

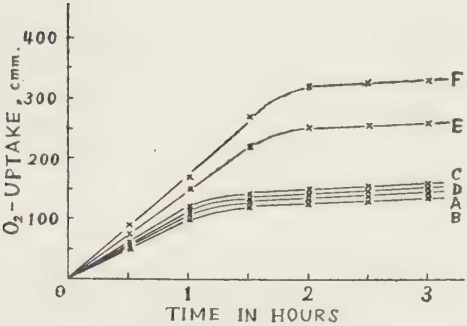

FIG. 1. Influence of Streptomycin on the oxidation of oxalacetate plus pyruvate. With the younger cells of *Mycobacterium tuberculosis avium* cultured for 5 days. The same results were obtained with the younger and the older cells. The vessel contents of the manometer were as follows.

Main chamber: 1.0 ml. of the cell suspension and 0.4 ml. of *M*/5 phosphate buffer solution (pH. 7.0).
Center well: 0.2 ml. of 20 per cent KOH (Dixon filter method).
Side arm: Substrates.
Streptomycin was added into the main chamber in 500 γ/ml.
The total volume was adjusted to 2.2 ml. with distilled water.
Water bath was regulated to 37°. After 30 minutes of preincubation, the content of the side arm was tipped into the main chamber.

A. 4 μM of oxalacetate.
B. 4 μM of pyruvate.
C. 4 μM of oxalacetate+Streptomycin.
D. 4 μM of pyruvate+Streptomycin.
E. 4 μM of oxalacetate+4 μM of pyruvate.
F. 4 μM of oxalacetate+4 μM of pyruvate+Streptomycin.

Effects of Various Antibiotics to C_4-Dicarboxylate Metabolism—The younger cells oxidize malate and its oxidation is hardly inhibited in the presence of a higher concentration of Streptomycin, as shown in Fig. 3. Succinate and fumarate give results as that of malate and the oxidations of pyruvate and acetate are not inhibited to any degree.

On the other hand, the older cells oxidize acetate, pyruvate, malate, fumarate, and succinate, and the oxidations of acetate and pyruvate are not inhibited as much as by the younger cells, but the oxidations of the others are remarkably inhibited in the presence of Streptomycin,

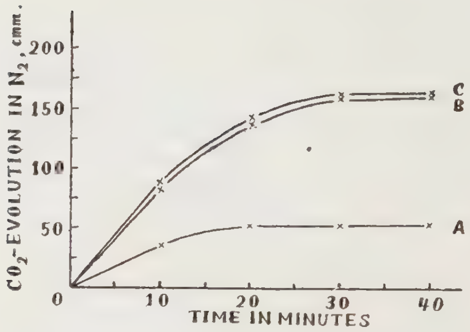

FIG. 2. Decarboxylation of oxalacetate by the cell-free extract of *Mycobacterium tuberculosis avium.*

The air in the vessel was exchanged with N_2-gas and CO_2-evolution measured without 20 per cent KOH. The other conditions were the same as those in Fig. 1.

A. 8 μM of oxalacetate, without the cell-free extract.
B. 8 μM of exalacetate with the cell-free extract.
C. 8 μM of oxalacetate and $10^{-4}M$ $MgCl_2$ with the cell-free extract.

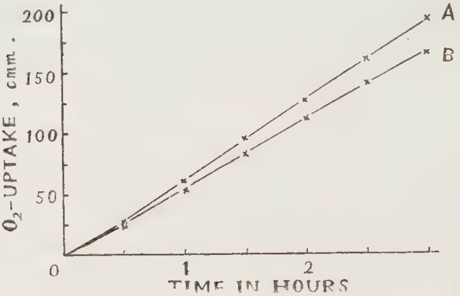

FIG. 3. Effect of Streptomycin on malate-oxidation of the younger (4 day-cultured) cell suspension.

The other conditions were the same as those in Fig. 1.

A. 20 μM of malate.
B. 20 μM of malate+500 γ/ml. Streptomycin.

as shown in Fig. 4. For example, the oxidation of succinate by the older cells is remarkably inhibited even in the presence of a low concentration of Streptomycin, as shown in Fig. 5.

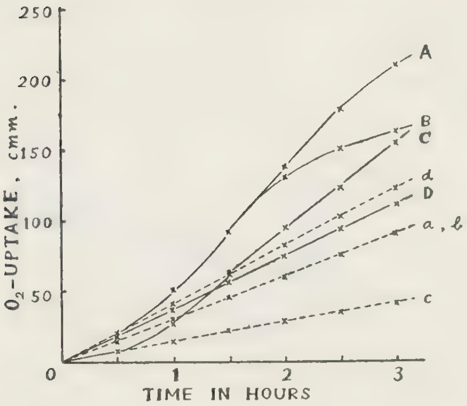

FIG. 4. Effect of Streptomycin on C_4-dicarboxylate-oxidation by the older cell suspension.

With the suspension of 7 day-cultured and 20 day-preserved cells. Streptomycin was added together with the substrates. The other conditions were the same as those in Fig. 1.

A. 8 μM of fumarate.
B. 8 μM of malate.
C. 8 μM of succinate.
D. 8 μM of pyruvate.
a. 8 μM of fumarate + 1000 γ/ml. Streptomycin.
b. 8 μM of malate + 100 γ/ml. Streptomycin.
c. 8 μM of succinate + 100 γ/ml. Streptomycin.
d. 8 μM of pyruvate + 100 γ/ml. Streptomycin.

The remarkable inhibition can be observed at 10 γ of Streptomycin per 15 mg. dry weight of the cells, which seems to equal the minimum concentration to inhibit the growth, and such an inhibition may be a cause for the growth inhibition.

Such inhibition of Streptomycin can not be demonstrated with streptomycin-resistant *Mycobacterium tuberculosis avium.* With the suspension of older cells resistant to 1000 γ of Streptomycin per ml., the oxidation of succinate is not inhibited in the presence of 25 to 100 γ of Streptomycin per ml. and little decreases in the rate at 500 γ per ml., as shown in Fig. 6.

The other antibiotics, Chloromycetin, Aureomycin, Flavomycin, and Terramycin, markly inhibit the oxidation of succinate by suspensions of the streptomycin-sensitive and -resistant cells, while Reticulin in-

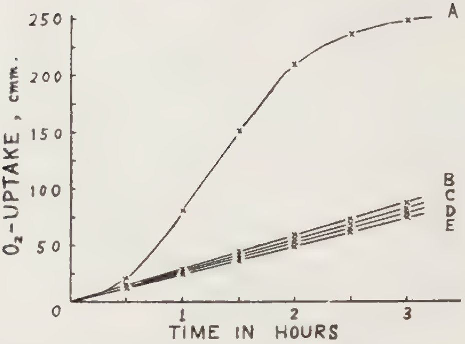

FIG. 5. Effects of Streptomycin on succinate-oxidation by the older cell suspension.

With the suspension of 7 day-cultured and 15 day-preserved cells. Streptomycin was added together with the substrates. Other conditions were the same as those in Fig. 1.

A. 8 μM of succinate.
B. 8 μM of succinate + 25 γ/ml. Streptomycin.
C. 8 μM of succinate + 50 γ/ml. Streptomycin.
D. 8 μM of succinate + 100 γ/ml. Streptomycin.
E. 8 μM of succinate + 500 γ/ml. Streptomycin

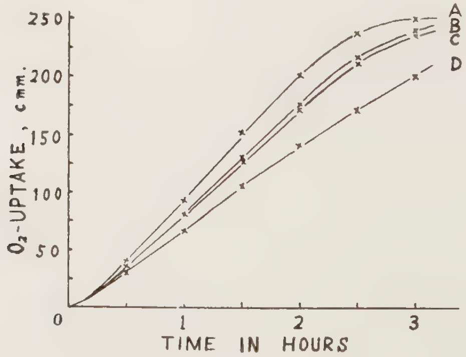

FIG. 6. Effect of Streptomycin on succinate-oxidation by the older cell suspension of the streptomycin-resistant substrain.

With the suspension of 7 day-cultured and 16 day-preserved cells. The cell suspension was preincubated for 45 minutes with Streptomycin before the addition of the substrates.

A. 8 μM of succinate.
B. 8 μM of succinate + 25 γ/ml. Streptomycin.
C. 8 μM of succinate + 100 γ/ml. Streptomycin.
D. 8 μM of succinate + 500 γ/ml. Streptomycin.

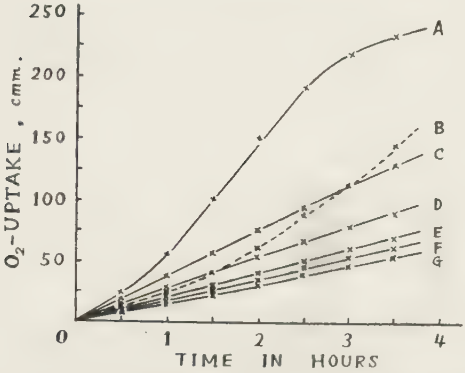

FIG. 7. Effect of various antibiotics on succinate-oxidation by the older cell suspension.

With the suspension of 7 day-cultured and 15 day-preserved cells. Antibiotics were added with succinate. Other conditions were the same as those in Fig. 1.

A. 8 μM of succinate.
B. 8 μM of succinate +100 γ/ml. Chloromycetin.
C. 8 μM of succinate +100 γ/ml. Reticulin.
D. 8 μM of succinate +100 γ/ml. Terramycin.
F. 8 μM of succinate +100 γ/ml. Flavomycin.
F. 8 μM of succinate +100 γ/ml. Streptomycin.
G. 8 μM of succinate +100 γ/ml. Aureomycin.

hibits the oxidation of succinate by the streptomycin-sensitive older cells, but not by the streptomycin-resistant cells as well as the inhibition of Streptomycin, as shown in Figs. 7 and 8.

It has been reported that attainment of reticulin-resistance runs parallel with that of streptomycin-resistance (*4*). It is, therefore, imaginable from such modes of action of these antibiotics that the inhibition of the dicarboxylate-oxidation by Streptomycin corresponds to the growth inhibition.

SUMMARY

1. The inhibition of the reaction between pyruvate and oxalacetate by Streptomycin as Umbreit has demonstrated with *Escherichia coli* is not demonstrable with *Mycobacterium tuberculosis avium.*

2. *Mycobacterium tuberculosis avium* has a strong power of oxalacetate-decarboxylation, and such a substance is extractable in a cell-

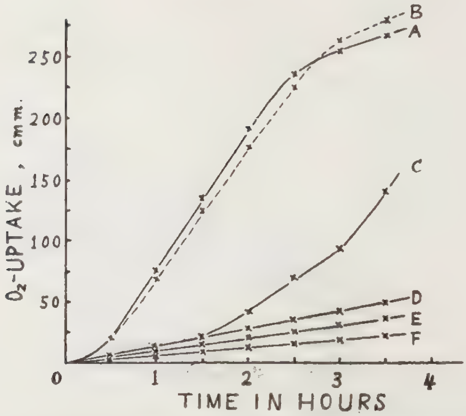

FIG. 8. Effects of various antibiotics on succinate-oxidation by the older cell suspension of the streptomycin-resistant substrain.

With the suspension of 7 day-cultured and 15 day-preserved cells. Other conditions were the same as those in Fig. 7.

A. 8 μM of succinate.
B. 8 μM of succinate +100 γ/ml. Reticulin.
C. 8 μM of succinate +100 γ/ml. Chloromycetin.
D. 8 μM of succinate +100 γ/ml. Aureomycin.
E. 8 μM of succinate +100 γ/ml. Flavomycin.
F. 8 μM of succinate +100 γ/ml. Terramycin.

free state. The cell-free extract decarboxylates oxalacetate in N_2-gas.

3. With the younger cells of *Mycobacterium tuberculosis avium*, both the streptomycin-sensitive strain and the resistant substrain, the oxidations of malate, fumarate, and succinate are hardly inhibited and the oxidations of pyruvate and acetate are not at all inhibited, in the presence of Streptomycin.

4. With the older cells of *Mycobacterium tuberculosis avium*, streptomycin-sensitive substrain, the oxidations of malate, fumarate, and succinate are remarkably inhibited in the presence of either Streptomycin, Aureomycin, Flavomycin, Terramycin, or Reticulin.

5. With the older cells of the resistant substrain, the oxidation of succinate is not inhibited in the presence of Streptomycin or Reticulin, while the oxidation is strictly inhibited by Chloromycetin, Flavomycin, Aureomycin and Terramycin.

REFERENCES

(1) Umbreit, W. W., *J. Biol. Chem.*, **177**, 703 (1949)

(2) Yamamura, Y., Kusunose, M., and Kusunose, E., *J. Biochem.* **39**, 227 (1952)

(3) Horio, T., and Okunuki, K., *J. Biochem.*, **41**, 709 (1954)

(4) Hosoya, S., Soeda, M., Komatsu, N., Okada, K., Sonoda, Y., and Arai, R., *J. Antibiotics (Japan)*, **5**, 251 (1952) (in Japanese)

The Journal of Biochemistry, Vol. 41, No. 6, 1954

STUDIES ON THE ACTION-PATTERN OF ANTITUBERCULAR SUBSTANCES

VI. ON THE MODE OF ACTION OF STREPTOMYCIN TO MYCOBACTERIUM TUBERCULOSIS AVIUM*

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(Received for publication, August 15, 1954)

In the previous investigation on the mode of action of various antibiotics to *Mycobacterium tuberculosis avium* (*1*), it was reported that the C_4-dicarboxylate-metabolism in the older cells of the streptomycin-sensitive strain is strictly inhibited in the presence of a minimum concentration of Streptomycin to prevent their growth and that such an inhibition may be the cause for its growth inhibition, although the inhibition of Streptomycin could not be demonstrated with the younger cells of *Mycobacterium tuberculosis avium.* The present investigations were then undertaken with the following notions. If some difference is distinguished between the younger and older cells, the point of action of Streptomycin will become more certain and some indication may be gained of a difference between the log-phase and lag-phase of the bacterial growth. By making use of the difficulty of *Mycobacterium tuberculosis avium* to autolyze, it was demonstrated that the ability to oxidize succinte shows rise and fall in the same cells and that such a phenomenon is inhibited in the presence of a low concentration of Streptomycin.

EXPERIMENTAL AND METHODS

Bacterial Strain and Culture—*Mycobacterium tubercurosis avium* grown on the glycerol buillon agar was used throughout this investigation.

Other Conditions and Methods—Refer to our previous report (*1*).

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* Our thanks are due to the Japan Autibiotics Association for financial support.

This was reported at the 25th General Meeting of Biochemical Society of Japan in Sendai, April, 1954.

RESULTS

The succinate oxidation by the older cell suspension of the streptomycin-sensitive strain is strictly inhibited in the presence of a low concentration of Streptomycin, but not at the time when the oxidation is proceeding at a maximum rate by the previous addition of the substrate, as shown in Fig. 1. This mode of action of Streptomycin is very similar to that against the formation of an adaptive enzyme of *Mycobacterium tuberculosis avium*, malonate-decarboxylase (*2*), and the enzymes to oxidize benzoate (*3*).

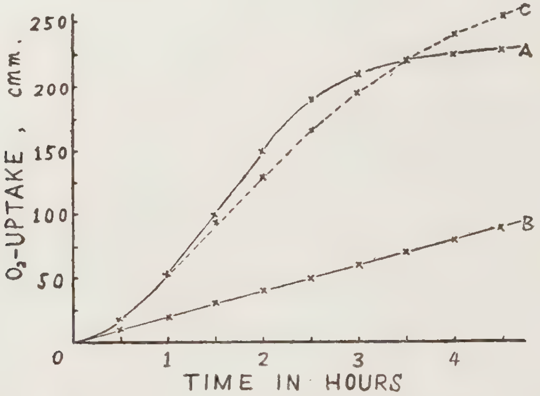

Fig. 1. Effect of Streptomycin on the succinate oxidation by the streptomycin-sensitive older cell-suspension.

The cells were suspended after 7 days' culture and 15 days' preservation. Each vessel of the manometer contained 1.0 ml. of the cell suspension (dry, wt. 16 mg./ml.) and 0.4 ml. of *M*/5 phosphate buffer solution (pH. 7.0), total volume adjusted to 2.2 ml. with distilled water. The water bath was regulated at 37°.

A. 8 μM of succinate
B. 8 μM of succinate+Streptomycin 25 γ/ml. added together with the succinate
C. 8 μM of succinate+Streptomycin 250 γ/ml. added one hour after the addition of the succinate.

There is however one difference between these adaptive enzymes and the succinate-oxidizing enzymes. In the younger cells of the streptomycin-sensitive strain, the succinate oxidation is not inhibited by Streptomycin so remarkably as in the older cells, as shown in Fig. 2.

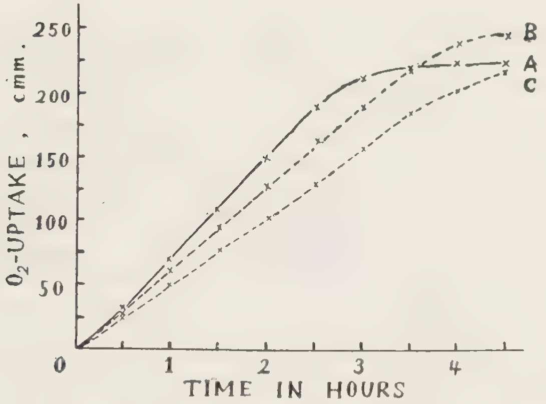

FIG. 2. Influence of Streptomycin on the succinate oxidation by the streptomycin-sensitive younger cell-suspension.

The cells were suspended immediately after 3 days' culture. The cell suspension (dry wt., 14 mg./ml.) was preincubated with Strepto mycin for half an hour before the addition of succinate. Other conditions were the same as those in Fig. 1.

A. 8 μM of succinate
B. 8 μM of succinate+Streptomycin 25 γ/ml.
C. 8 μM of succinate+Streptomycin 250 γ/ml.

This phenomenon resembles the glycerol oxidation previously reported (*4*).

On the other hand, the formation of the adaptive enzymes is strictly inhibited in the presence of Streptomycin in the younger as well as in the older cells. These results indicate that the younger cells possess constitutively the so-called succinate-oxidizing enzymes to the full, while the older cells possess only a small amount of the enzymes and the enzymes are adaptively formed on the addition of the substrate. As shown in Table I, the inhibitions of Streptomycin and of Reticulin are observed only with the older cells of the streptomycin-sensitive strain, and Chloromycetin, Aureomycin, Terramycin and Flavomycin inhibit strictly the succinate oxidations with the older cells of the streptomycin-sensitive and -resistant substrains. In culture experiments, resistance against Reticulin is known to be attained parallel to that against Streptomycin (*5*). Hence the result of Table I shows a specificity of Streptomycin and suggests that such inhibition of Streptomycin is a cause in preventing the bacterial growth.

TABLE I

Effects of Various Antibiotics on Succinate Oxidation by the Younger and Older Cells of the Streptomycin-sensitive Strain and the Streptomycin-resistant Substrains

Antibiotics	% of Inhibition on succinate oxidation			
	Streptomycin-sensitive strain		Streptomycin-resistant substrain	
	Younger Cells	Older cells	Younger cells	Older cells
Streptomycin	14	70	2	4
Reticulin	9	50	2	5
Chloromycetin	11	52	26	86
Aureomycin	25	76	33	91
Terramycin	24	67	34	94
Flavomycin	22	71	29	97

The younger cells, 3 days' culture. The older cells, 7 days' culture and 26 days' preservation. Each antibiotic was added to the final concentration of 100 γ/ml. together with succinate. Other manometric conditions were the same as those in Fig. 1. Arabic numerals express per cent of inhibition, which was calculated at a point where the succinate oxidation without antibiotics reached equillibrium.

In order to demonstrate that the so-called constitutive enzymes to oxidize succinate increase and decrease in the same cells, the older cells of the streptomycin-sensitive strain were treated as follows: Each of three flasks, FA, FB, and FC, contained 10 volumes of the older cell suspension of the streptomycin-sensitive strain and 4 volumes of $M/5$ phosphate buffer solution (pH 7.0). Two volumes of $M/25$ succinate aq. was added to FB and FC. Immediately after the preparation, the experiments shown in Fig. 3 was carried out using a portion of the mixture of FA. At the same time, the three flasks were shaken at 37°. After 2 hours, 2 volumes of Streptomycin aq. was added into FC to a final concentration of 10 γ per ml. After 4 hours of the shaking, the experiment shown in Fig. 4 was carried out, using a portion separated from FB. After 10 hours of the shaking, the experiments shown in Figs. 5 and 6 were carried out with FA, FB, and FC. These results are summarized in Table II. At first, the succinate oxidation suffers 60 per cent inhibition in the presence of 10 γ of Streptomycin per ml. (see FA_0-

TABLE II

Oxidations of Succinate and Acetate by the Streptomycin-sensitive Older Cell-Suspension Shaken at 37°

Flasks used	Shaking hours, hrs	Succinate oxidation			Acetate oxidation				
		Inhibition of Streptomycin (STM), %	Oxygen atoms per mole of succinate		Oxidation-rate, minutes		Inhibition of STM %	Oxygen atoms per mole of acetate	
			non STM	STM	non STM	STM		non STM	STM
FA	0	60	2.3	—	33	33	0	2.2	2.2
FB	4	9	—	—	—	—	—	—	—
FA	10	—	—	—	33	34	1	2.5	2.5
FB	10	40	3.2	—	—	—	—	—	—
FC	10	-2	—	4.2	—	—	—	—	—

Rate of acetate oxidation is expressed by time in minutes required for the oxygen-uptake to arrive at 100 cmm. Per cent of streptomycin inhibition was calculated at the point where the succinate oxidation without Streptomycin reached 150 cmm. of oxygen-uptake. For experimental conditions, cf. Figs. 3, 4, 5 and 6.

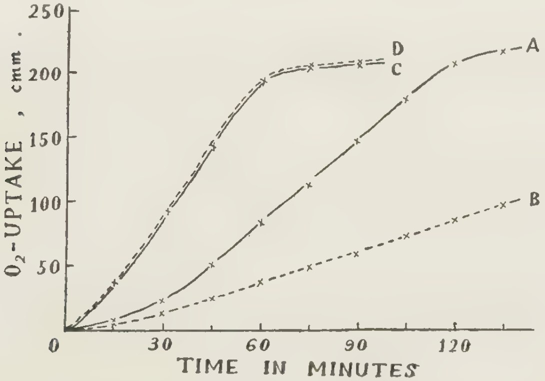

FIG. 3. Effect of Streptomycin on the succinate and acetate oxidation by the streptomycin-sensitive older cell-suspension.

With the suspension (dry. wt., 24 mg./ml.) of 7 days' culture and 10 days' preserved cells. Other manometric conditions were the same as those in Fig. 1. This experiment was carried out with a portion of FA.

A. 8 μM of succinate
B. 8 μM of succinate + 10 γ/ml. Streptomycin
C. 8 μM of acetate
D. 8 μM of acetate + 100 γ/ml. Streptomycin

Streptomycin was added together with succinate and acetate.

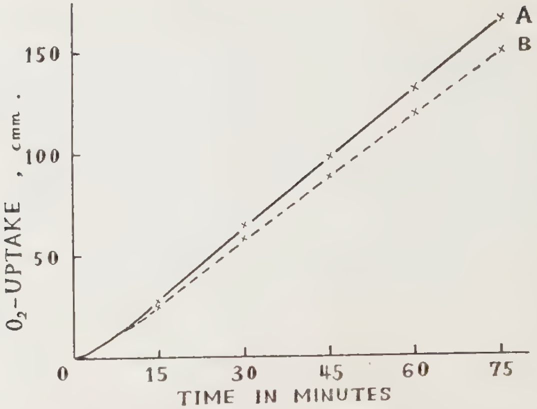

FIG. 4. Effect of Streptomycin on succinate oxidation by the streptomycin-sensitive older cell-suspension shaken with succinate for 4 hours.

This experiment carried out with a portion of FB. Other experimental conditions were the same as those in Fig. 3.

A. 8 μM of succinate

B. 8 μM of succinate+100 γ/ml. Streptomycin

$_{\text{hr.}}$). This marked inhibition decreases to only 9 per cent in the cell suspension shaken with the succinate during 4 hours in the presence of 100 γ of Streptomycin per ml. (see $FB_{4\,\text{hr.}}$). If the same suspension is shaken more, the inhibition of Streptomycin gradually appears again, and the inhibition increase again to 40 per cent after 10 hours (see $FB_{10\text{-hr.}}$). With the cell suspension shaken with succinate and Streptomycin, no inhibition is observed even after 10 hours (see $FC_{10\,\text{hr.}}$). On the other hand, acetate is oxidized at the same rate in the shaken and non-shaken cell-suspensions (see $FA_{0\,\text{hr.}}$ and $FA_{10\,\text{hr.}}$). This indicates that the cells hardly suffer autolysis but only the loss (changeé) of intracellular materials by the long shaking.

DISCUSSION

In the previous report (*4*), influence of various inhibitors and antibiotics on the metabolism of glycerol, important as the carbon source in the culture of *Mycobacterium tuberculosis*, was studied and it was shown that Streptomycin influences more strict inhibition on the glycerol metabolism of the older than the younger cells. The glycerol-oxidizing

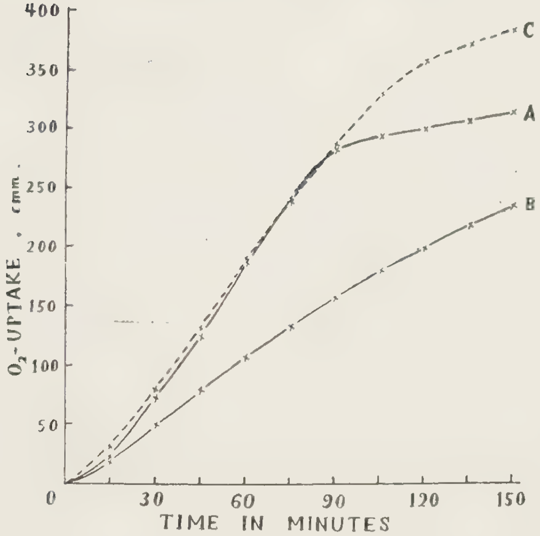

FIG. 5. Effect of Streptomycin on the succinate oxidations by the streptomycin-sensitive older cell-suspension shaken with succinate and by that shaken with succinate and streptomycin, for 10 hours.

These experiments were carried out with a portion from FB and FC. Other conditions were the same as those in Fig. 3. Curves A and B, from flask FB, Curve C, from flask FC.

A. 8 μM of succinate
B. 8 μM of succinate+10 γ/ml. Streptomycin
C. 8 μM of succinte+20 γ/ml. Streptomycin (10 γ/ml. Streptomycin was added afresh).

enzymes which appear to carry out some important role in the growth of *Mycobacterium tuberculosis* are likely to be formed adaptively for the most part in such cells as are carrying out only the endogeneous respiration, and the formation is likely to be inhibited in the presence of Streptomycin. In fact, Streptomycin strictly inhibits the formations of adaptive enzymes of *Mycobacterium tuberculosis avium*, malonate-decarboxylase (*2*), and benzoate-oxidizing enzymes (*3*), as well as adaptive enzyme formations of the other species of bacteria (*6*).

Mycobacterium tuberculosis does not form spores. Regardless of sporulation, however, it is difficult to conjecture that all of the enzymes existing in a cell on log-phase, the so-called constitutive enzymes, are carrying out their functions fully even in the cells in lag-phase. So far as

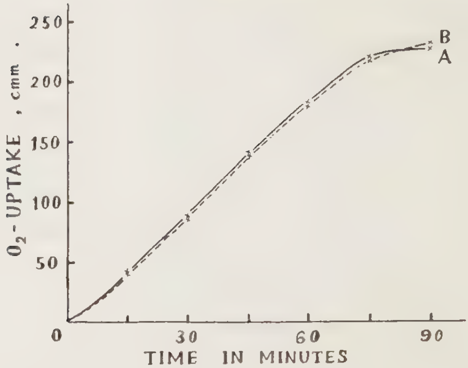

FIG. 6. Effect of Streptomycin on acetate oxidation by the streptomycin-sensitive older cell-suspension shaken for 10 hours.

This experiment was carried out with a portion of FA. The experimental conditions were the same as those in Fig. 3.

A. 8 μM. of acetate

B. 8 μM. of acetate + 100 γ/ml. Streptomycin

enzymes are concerned, there are two possibilities of the metabolism in a cell on lag-phase.

(a) Some substances competitively inhibit the already existing enzyme systems. For example, malonate (*2*, *7*) or canavanosuccinate (*8*), which itself is metabolizable, competitively inhibits succinic dehydrogenase or arginosuccinase. Malonate can be an intermediate of nucleic acid and canavanosuccinate from protein ; (b) The enzymes of a cell on log phase, the so-called constitutive enzymes, are changed into non-functional substances until only the enzymes necessary for endogeneous respiration are left.

The C_4- dicarboxylate metabolism of *Mycobacterium tuberculosis avium* has been studied and it demonstrated that the cells in lagphase have far less succinate-oxidizing enzymes than those in log-phase and that the amount of the enzymes rises and falls. From these results, it may be concluded that the cells on log-phase are full of the enzymes essential for growth and proliferation, *e.g.*, enzymes connected with TCA-cycle or the glycerol oxidizing enzymes, and as the cells become older, perhaps on account of the lack of nutrient, area to spread, or the fall of temperature, *etc.* in the test-tube experiments, more than one part of the enzyme-systems is metabolized till almost only the endogeneous respiration is left. Other results indicate that Streptomycin inhibits the formation and degradation of the succinate-oxidizing enzymes, the so-called constitutive enzymes, as well as the adaptive enzymes of *Mycobacterium tuberculosis avium.* Generally speaking, Streptomycin may inhibit formation and degradation of enzymes, whether those are constitutive or adaptive, and enzymes will be fixed as they are and microorganisms will fail to proliferate, by the addition of Streptomycin. This seems to be the essence of the bacteriostatic action of Streptomycin. The streptomycin-resistant cells of *Mycobacterium tuberculosis avium* suffer no influence of Streptomycin on the so-called constitutive as well as the adaptive enzyme formation. In other words, the streptomycin-resistant cells are likely to be able to form some enzymes of the same function as of the enzymes the streptomycin-sensitive cells cannot form, in the presence of Streptomycin. Acquisition of Streptomycin resistance may mean the ability to form enzymes in the presence of Streptomycin.

SUMMARY

1. With the streptomycin-sensitive older cells of *Mycobacterium tuberculosis avium*, the oxidation of succinate is remarkably inhibited in the presence of a low concentration of Streptomycin, but not in the presence of a higher concentration of it as long as the cells are full of the succinate oxidizing enzymes by the previous addition of a substrate.

2. Streptomycin and Reticulin markedly inhibit the succinate oxidation of only the older cells of the streptomycin-sensitive strain of *Mycobacterium tuberculosis avium*, while Aureomycin, Terramycin, Flavomycin, and Chloromycetin markedly inhibit the succinate oxidation of older cells, whether those are sensitive or resistant against Streptomycin.

3. In *Mycobacterium tuberculosis avium*, the ability to oxidize succinate rises and falls in response to the presence and absence of the substrate. Such rise and fall in the streptomycin-sensitive cells is inhibited in the presence of a low concentration of Streptomycin, but not in the streptomycin-resistant cells.

REFERENCES

(*1*) Horio, T., and Okunuki, K., *J. Biochem.* **41**, 725 (1954)
(*2*) Horio, T., and Okunuki, K., *J. Biochem.*, **41**, 717 (1954)
(*3*) Yamamura, Y., Sasagawa, T., and Adachi, T., *I Ryo*, (*Tokyo*), **3**, 17 (1949) (in Japanese)

(*4*) Horio, T., and Okunuki, K., *J. Biochem.*, **41**, 709 (1954)

(*5*) Hosoya, S., Soeda, M., Komatsu, N., Okada, K., Sonoda, Y., and Arai, R., *J. Antibiotics* (*Japan*), **5**, 251, (1952)

(*6*) Suda, M., and Naono, S., *Med. J. Osaka Univ.*, **3**, 95 (1952)

(*7*) Yamada, K., and Suzuki, T., *J. Agr. Chem. Soc. Japan*, **25** 290, 372 (1951)

(*8*) Water, J. B., *J. Biol. Chem.*, **204**, 139 (1953)

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NITRATE REDUCTION BY CL. WELCHII

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Many bacteria are known to be able to reduce nitrates to nitrites. In some facultative anaerobes, nitrate enables them to grow anaerobically, *i. e.* nitrate is able to take the place of molecular oxygen (*1*, *2*). The reduction of nitrates by *E. coli* has been extensively studied by several workers (*3–7*), and the utilization of nitrate as a hydrogen acceptor was shown to depend on the ability of the organism to activate the nitrate molecule, that is, the activity of nitrate reductase (*5*). Recently, Sato, Niwa and one of us (Egami) (*8*), using a cell-free enzyme preparation, have deduced that the nitrate reductase of *E. coli* appears to be an iron-containing enzyme. On the other hand, according to Nicholas, Nason and McElroy (*9*), the nitrate reductase of *Neurospora* is a molybdenum-containing enzyme.*

In 1938, Woods (*10*), in the studies on the enzymic make up of washed cells of *Cl. welchii*, one of the well known strict anaerobes, confirmed the presence of hydrogenase and showed that nitrate, nitrite, and hydroxylamine were reduced quantitatively to ammonia by molecular hydrogen by the organism. It seemed interesting to us to study the reduction of nitrates by *Cl. welchii* comparing it with that by *E. coli* from the view point of elucidating the chemical nature of the nitrate reducing systems. The present paper deals with a preliminary survey of nitrate reduction by the washed cell suspension, presenting evidences of the presence of nitrate reductase in *Cl. welchii*. Some of the properties of the nitrate reducing activity of the organism will be discussed.

The outline of this work was read at the general meeting of the Japanese Biochemical Society held at Kobe, in May, 1952 (*11*).

* According to a private communication from W. D. McElroy, nitrate reductase of a luminous bacterium seems to be an iron enzyme.

METHODS AND MATERIALS

The strain of *Cl. welchii* used in this work was the PB-6K strain, supplied from the National Institute of Health of Japan. This was cultivated anaerobically on the liver broth medium containing 0.1 per cent glucose and 0.1 per cent KNO_3, for 14 to 16 hours at 37°. The culture was centrifuged and the cells were washed three times with cold, *M*/15 phosphate buffer solution of pH 7.0 in the refrigerated centrifuge.

The cells were finally suspended in the buffer to give a concentration of about 2 mg. bacterial N per ml (turbidimetrically determined). The cell suspension obtained retained its enzyme activity for about 2 days when kept anaerobically at about 0°. The course of the reduction of nitrate was followed by keeping the suspension anaerobic in the Thunberg tube in the presence of a known concentration of nitrate (0.001 *M*) and of hydrogen donator (mainly glucose) and estimating the nitrite produced colorimetrically by means of the Griess-Ilosvay reagent as described by Stickland (*4*) and also by Egami and Sato (*6*). Dehydrogenating activity of the cells was checked by measuring decolorization time of a given amount of methylene blue added.

The method of colorimetric determinations of nitrite formed: A portion of the reaction mixture (2 ml.) was taken, added with a half volume of saturated solution of uranyl acetate to precipitate protein and to stop the enzyme action, and with a small amount of active charcoal to remove the dye. One or two ml. of clear filtrate was added with 2.5 ml. of Griess-Ilosvoy reagent and made up to 10 ml. with 50 per cent acetic acid. The intensity of colour developed in 15–20 minutes was estimated by means of Pulfrich Stufenphotometer (filter S_{53}). All determinations were carried out in duplicate.

EXPERIMENTAL

Extent of Nitrate Reduction by Washed Cells of Cl. Welchii—It is known that the reduction of nitrate to nitrite by *E. coli* takes place as a result of a coupled reduction with dehydrogenation of some hydrogen donators (*e.g.* lactate, formate). Our preliminary experiments showed that washed cells of *Cl. welchii* has a strong activity of reducing nitrate to nitrite, particularly in the presence of glucose or ethyl alcohol as a hydrogen donator. The results of experiments using various substances as hydrogen donators are shown in Table I. Dehydrogenation was necessary to bring about nitrate reduction, the approximate rate of which was estimated by the decolorization time of methylene blue added. It is known that in the case of *E. coli* methylene blue behaves as an intermediary hydrogen carrier in the nitrate reduction and complete decolorization is not attained even in the presence of sufficient amounts of hydrogen acceptors owing to the recoloration by nitrate (*5, 6*). But this was not the case with the nitrate reduction by *Cl. welchii*, where complete decolorization was always seen even in the presence of a large amount of nitrate. This point will be discussed later. With a longer period of the reaction, nitrate was found to be reduced almost completely with the coupled dehydrogenation of glucose, as shown in Fig. 1. Although with

TABLE I

Nitrate Reduction of Cl. welchii, Coupled with Dehydrogenation of Various Hydrogen Donators

Donator	Dehydrogenation (decolorization of methylene blue)	Nitrate reduction (formation of nitrite)
− (control)	−	−
Glucose	++ (2′)	++
K formate	−	−
Na acetate	−	−
Na succinate	−	−
Na lactate	−	−
Na citrate	−	−
Na malate	+ (50′)	+
Na glutamate	−	−
L-Alanine	−	−
Na gluconate	± (100-120′)	−
Na pyruvate	+ (15-30′)	+
Ethanol	+ (4′)	+
Glycerol	+ (50′)	+
Acetaldehyde	+ (17′)	+

Donator: 100 μM.

Cell suspension: 0.2 ml. (Final concentration of cells: 0.05 mg. bacterial N per ml.).

Buffer: 3 ml. of $M/15$ phosphate, pH 7.0.

Acceptor: 0.2 ml. of 0.1 per cent methylene blue and 1 ml. of 0.01 M KNO_3.

Total volume: 10 ml. Temperature; 40°.

The sign (−) denotes a negative result of reduction when estimated at 120 minutes reaction time. The sign (+) shows a positive result of reduction, where decolorization time of methylene blue is noted in parenthesis. Formation of nitrite was determined 20–30 minutes after decolorization of methylene blue.

a thicker suspension of cells the slow reduction of nitrite formed was observed, we were able to differentiate the reduction of nitrate to nitrite from the further nitrite reduction by taking lower concentrations of cells and shorter reaction periods, where the latter process was sufficiently minimized. The linearity of the initial course of the reaction (nitrate→nitrite) with varying cell concentrations and donator concentrations was demonstrated respectively, within the limits of the accuracy of the method, in Fig. 2-a and 2-b.

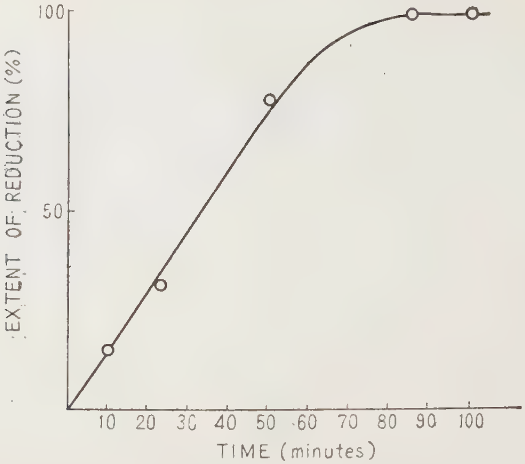

FIG. 1. Whole course of reduction of nitrate to nitrite by *Cl. welchii.* Hydrogen donator: 20μM glucose. Hydrogen acceptor: 10 μM KNO_3. Concentration of cells: 0.1 mg. bacterial N per ml. Total volume of reaction mixture: 10 ml. pH: 7.0. Temp., 40°.

As is seen from Fig. 2-c. there is no great decrease in velocity of nitrate reduction with diminishing concentrations of nitrate within the range of experimental conditions.

FIG. 2-a FIG. 2-b FIG. 2-c

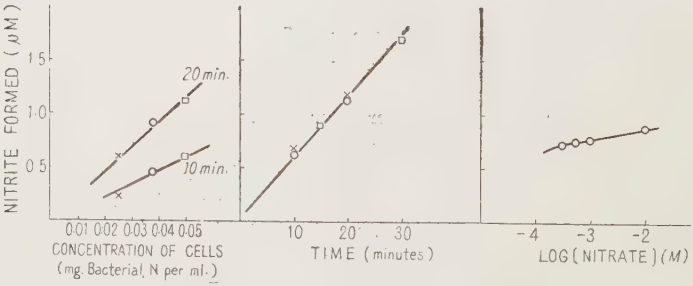

FIG. 2-a. Glucose 20 μM. KNO_3 10 μM.
FIG. 2-b. Amount of glucose. × (500 μM). ○ (50 μM). □ (20 μM).
Amount of KNO_3 10 μM.
Concentration of cells: 0.05 mg. bact. N per ml.
FIG. 2-c. Glucose 20 μM.
Concentration of cells: 0.05 mg. bact. N per ml.

The optimum pH for the nitrate reduction was found to be within pH 6.5 to 7. (Fig. 3).

The stable pH range of the enzymic activities for overall reactions was 6.0 to 7.0. (Fig. 4)

FIG. 3

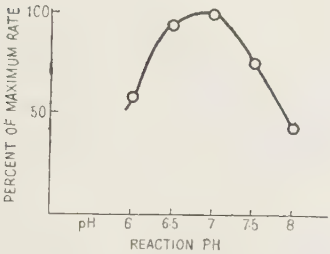

FIG. 4

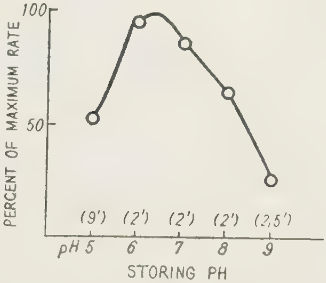

FIG. 3. In keeping various pH values, phosphate and borate buffer were used. Dehydrogenation of glucose tested by decolorization of methylene blue added was found to have nearly the same activity throughout the pH range tested (decolorization time of methylene blue, 2 minutes).

FIG. 4. Cell suspensions kept at various pH at about 0° for 12 hours were tested for their reducing activities of nitrate and methylene blue at pH 7.0. Reduction of nitrate was expressed in % of maximum rate and the decolorization time of methylene blue was shown in parentheses above the storing pH values noted in the figure.

Effect of Redox Indicators—In the case of *E. coli*, it was shown that the redox indicator such as methylene blue served as an intermediary hydrogen carrier *in vitro*. Thus, leuco-methylene blue prepared by the catalytic hydrogenation of methylene blue with Pd-$BaSO_4$ as a catalyst can be used as a hydrogen donator for the reduction of nitrates, enabling us to study the nitrate reduction apart from the dehydrogenase systems. But, *Cl. welchii* cells can completely decolorize methylene blue even in the presence of a large amount of nitrate, and leucoform of this dye was found not to be used as a hydrogen donator. Moreover, methylene blue in excess showed inhibiting effect on the nitrate reduction by *Cl. welchii* cells as shown in Fig. 5. The effects of several indicators of decreasing redox potentials such as Nile blue, Janus red (prepared by the hydrogenation of Janus green with Pd-$BaSO_4$) and neutral red were tested. It was found that neutral red whose normal redox potential was as low as -0.34 volt (*16*) could serve as an intermediary hydrogen carrier. It was decolorized in the absence of nitrate and recolorized by adding nitrate as a hydrogen acceptor. The presence of neutral red in the reaction mixture tends to activate nitrate reduction by the cell suspension*.

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* The action of leuco form of Janus red as a hydrogen donator was shown often effective for nitrate reduction but the results were inconsistent.

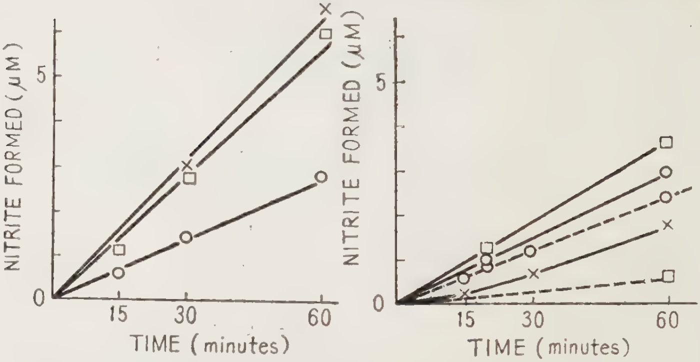

Fig. 5. *E. coli* (0.02 mg. N per ml.) Hydrogen donator HCOOK 20 μM ○ without dye × with 0.5 ml. of 0.1% MB □ with 3 ml. of 0.1% MB

Cl. welchii (0.02 mg. N per ml.) Hydrogen donator: Glucose 20 μM ○---○ without dye ×—× with 0.5 ml. of 0.1% MB □---□ with 1 ml. of 0.1% MB ○—○ with 2 ml. 0.05% Neutral red □—□ with 3ml. 0.05% Neutral red

Table II

Effect of Addition of Nitrate to Culture Medium

	Cells		
	Control (without addition of nitrate)	0.02%* KNO_3	0.1%* KNO_3
Decolorization time of methylene blue	2′	2′	2′
Nitrate reduction	Nitrite Formed μM		
Time 15′ 30′	 0.75 1.27	 1.3 2.5	 2.0 3.9

* Concentration of nitrate in culture medium.

Hydrogen donater; 20 μM Glucose.

Hydrogen acceptor: 10 μM KNO_3 and 0.2 ml. of 0.1 per cent methylene blue.

Cell concentration: 0.1 mg. bacterial N per ml.

Total volume: 10 ml. pH: 7.0. Temp. 40°.

Effect of Addition of Nitrate to Culture Media on the Nitrate Reducting Activity—The addition of nitrate to culture medium was found to promote the nitrate reducing activity of *Cl. welchii* without any effect on the dehydrogenating activity as shown in Table II, the fact suggesting apparently adaptive nature of the nitrate reductase. Our materials used throughout these investigations were derived from the culture medium containing 0.1 per cent nitrate.

Effects of Some Inhibitors on the Nitrate Reducing Activity—Using mainly the vacuum tube method for measuring the reaction rates, the effects of some inhibitors on the nitrate reducing activity of the washed cell suspension were examined. In these cases, it was necessary to test the effects of inhibitors on the dehydrogenase systems, which would evidently influence the rate of nitrate reduction. These effects were determined by comparing decolorization time of a given amount of methylene blue added simul-

TABLE III

Effect of Some Inhibitors on Nitrate Reduction

Inhibitor	concentration	Effect on nitrate reduction degree of inhibition
	M	%
KCN	10^{-2}	100
	10^{-3}	90
	10^{-4}	66
Monoiodoacetate	10^{-3}	100
	10^{-4}	50
Thiourea	10^{-2}	70
	10^{-3}	52
	10^{-4}	25
Cysteine	10^{-3}	30
Thioglycolate	10^{-3}	33
Ascorbic acid	10^{-3}	30
NaF	10^{-2}	0
CO	1 atom	0

Hydrogen donator: 100 μM glucose, Acceptor: 10 μM KNO_3.

Concentration of cells: 0.05 mg. bacterial N per ml.

One ml. of inhibitor solution was added in the side room of the Thunberg tube with 1 ml. of 0.01 *M* KNO_3 and 0.2 ml. of 0.1 per cent methylene blue.

Total volume: 10 ml. pH 7.0; Temp. 40°.

Absence of the effect of inhibiting agents except cysteine on the determination of nitrite was previously confirmed. A corrected value was shown in the experiment with cysteine ($10^{-3}M$), which caused some inhibition on the colorimetric determination of nitrite.

TABLE IV

Hydrogen Uptake and Effect of Cyanide

Systems 2.5 ml. pH 7					
	Cell : 1 mg. bacterial N per ml. (in $M/15$ pbosphate buffer, pH 7)	2 ml.	2 ml.	2 ml.	2 ml.
	KNO_3	0.5 ml. (0.01 *M*)	0.25 ml. (0.02 *M*)	—	—
	KCN	—	0.25 ml. (0.01 *M*)	—	0.35 ml. (0.007 *M*)
	Methylene blue	—	—	0.15 ml. (1%)	0.15 ml. (1%)
	Aqua	—	—	0.35 ml.	—
Hydrogen uptake in 30 min. in μl.		197	24.5	114	110

H_2 uptakes were measured in Warburg manometers at 37°.
0.2 ml. of 10 per cent NaOH was placed in the inner tubes as CO_2 absorber.
The manometers were filled with H_2 purified by passage over heated copper.

TABLE V

Nitrate Reduction by Cells of Varying Concentrations of Iron

Cells			Activity of reduction†	
Iron concentration in medium (γ per ml.)	Growth (mg. bact. N. per ml.)	Iron concentration in cells (γ per mg. bact. N)	Decolorization time of MB (minute)	Nitrite formed μM
0.6	0.040	17	2	3.8
0.13	0.046	3.5	2–3	3.3
0.03	0.028	1	8	1.0

† Hydrogen donator: 100 μM Glucose. Concentration of cells: 0.1 mg. bacterial N per ml. Amount of nitrite formed was estimated at 20 minutes reaction time. Iron was determined by the α,α'-dipyridyl method (*17*).

taneously with nitrate in the reaction mixture with that found in control tubes containing no inhibitor. Table III summarized the degree of inhibition of nitrate reduction exhibited by several agents in concentrations noted, which showed no inhibitory effects on dehydrogenation of glucose (methylene blue as an acceptor).

Nearly the same degree of inhibition by cyanide was demonstrated on the nitrate reduction by *Cl. welchii* as on that by *E. coli* enzyme (*4*, *8*), that is, about 50 per cent inhibition at 10^{-4} *M*, and almost complete inhibition at 10^{-3} *M* KCN. Woods (*10*) studied the nitrate reduction of *Cl. welchii* by coupling with the hydrogenase action, using the manometric technique. In our experiment shown in Table IV the absorption of hydrogen by the washed cells in the presence of methylene blue as an acceptor was found not to be affected by 10^{-3} *M* CN^-, but, when nitrate was substituted for methylene blue as an acceptor, it was inhibited as much as 90 per cent in the presence of the same concentration of cyanide. This offered an evidence of the presence of the nitrate-activating enzyme distinct from hydrogenase, the activity of which appeared to be sensitively affected by cyanide.

Monoiodoacetate, thiourea, cysteine, thioglycolate, and ascorbic acid, all of which inhibit nitrate reductase of *E. coli*, also showed marked inhibition on the enzyme activity of *Cl. welchii* without any appreciable effects on the dehydrogenating activities. Fluoride showed no inhibitory effects both on dehydrogenation of glucose and on nitrate reduction.

It must be pointed out here that the inhibitory action of CO on the nitrate reductase of *E. coli* and its reversible recovery by light reported by Sato and Egami (8) was not observed with that of *Cl. welchii* cells.

α,α'-Dipyridyl (10^{-3}, 10^{-4} *M*) was shown to have inhibitory effects at pH 6.4 on dehydrogenating activities on glucose and ethyl alcohol and also on nitrate reducing activity.

Presence of oxygen or toluene was found to prevent completely the formation of nitrite from nitrate.

DISCUSSION

Our experimental results showed that the washed cell suspension of *Cl. welchii* grown in the culture medium containing 0.1 per cent glucose and 0.1 per cent KNO_3 strongly reduced nitrate to nitrite coupling with dehydrogenation of glucose or ethyl alcohol, as well as with a hydrogenase system in the presence of molecular hydrogen. In these reactions, the presence of nitrate reductase as distinct from dehydrogenases or hydrogenase in the cells of *Cl. welchii* was clearly confirmed from the following facts:

(1) The addition of nitrate in culture media caused a marked increase of nitrate reducing activity of the organism, without any effects on the dehydrogenating activity of glucose of the cells;

(2) Several inhibitors such as cyanide, monoiodoacetate and thiourea etc. caused pronounced inhibition of the formation of nitrite from nitrate, with no appreciable effect on the dehydrogenase activities ;

(3) The manometric experiments showed that cyanide ($10^{-3}M$) caused strong inhibition on the absorption of hydrogen in the presence of nitrate as a hydrogen acceptor, while no inhibitory effect was seen when methylene blue took the place of nitrate as a hydrogen acceptor.

From these results it may be concluded that cells of *Cl. welchii*, like those of *E. coli*, contain the distinct enzyme which participates in the reduction of nitrate and that nitrate without such enzymatic activation cannot accept hydrogen through dehydrogenases or hydrogenase systems.

The characteristic feature concerning reduction of nitrate by *Cl. welchii in vitro* is that only the redox indicator of sufficiently low potential such as neutral red has been found effective as an intermediary hydrogen carrier in this reaction. It seems to indicate that the nitrate reductase of *Cl. welchii* has a lower normal potential than that of *E. coli*. It may be related to the fact that strict anaerobes demand the medium with lower redox potential for their growth. Methylene blue, a known intermediate hydrogen carrier *in vitro* for the nitrate reductase of *E. coli*, could not play its role in the reduction of nitrate by *Cl. welchii*, and appeared to compete with nitrate as an ultimate oxidant inhibiting the reduction of nitrate.

Thus it seems that in *Cl. welchii* cells, the hydrogen transferring route may branch in two ways at some intermediate towards methylene blue and nitrate respectively, and may be devoid of the pathway from the leucomethylene blue to nitrate. A similar relation was observed recently with the sulfate reduction by *D. desulphuricans* (*18*). Analysis of natural hydrogen transferring system in nitrate reduction of such anaerobic cells as *Cl. welchii*, in connection with that of *E. coli* (*6, 8*), may deserve further investigation.

In view of the effects of several inhibitors on nitrate reductase, there seems to be no distinct differences between *E. coli* and *Cl. welchii*, except the effect of carbon monoxide as stated above. Sato and Egami (*8*), in studying the partially purified enzyme preparation of *E. coli*, suggested that the nitrate reductase might be an iron-containing enzyme. Smith *et al.* (*12*) in studying heme utilisation by *H. influenzae*, assumed that its nitratase (nitrate reductase) might be a heme enzyme. In order to survey any role of iron in nitrate reduction by *Cl. welchii*, we prepared iron-deficient cells by cultivation on the iron-poor, casein hydrolysate medium described by Pappenheimer and Shaskan (*13*). Removal of iron from the medium was carried out by an 8-hydroxyquinoline-chloroform extraction method according to the procedure of Waring and Werkman (*14*). Both the dehydrogenating activity for glucose and the nitrate reductase activity of the washed cells of lower iron content was found to be considerably decreased, compared with those of cells containing higher amounts of iron, as shown in Table V. Since the extent of the overall reaction of reduction decreased in iron-deficient cells, no decisive proof of the essential role of iron on the nitrate reductase system itself was obtained from the experiment. *α,α'*-Dipyridyl also showed inhibitory effect on reduction of both methylene blue and nitrate. (In this connection Bard and Gunsalus (*15*) studied glycolysis of iron-deficient *Cl. welchii* showing the essentiality of iron for the function of aldolase.) So far as we are dealing with the coupled reduction of nitrate with dehydrogenation of glucose or with hydrogenase, the problem seems too complex to clarify the chemical nature of the nitrate reducing enzyme. The establishment of a method of determining enzyme activity apart from dehydrogenases or hydrogenase systems will be necessitated. Anyhow the effects of inhibitors seem to indicate the contribution of some heavy metal to the enzyme system.

In several attempts to obtain the active enzyme preparation in a cell-free state using various techniques of extraction, such as freezing and thawing, ultrasonic disintegration procedures *etc.*, we have recently succeeded in obtaining it by grinding young cells harvested after about 6 hours cultivation by means of Potter's homogenizer with a small amount of alumina powder in the cold, followed by a high speed centrifugation, but only with a low yield.

SUMMARY

Reduction of nitrate to nitrite by washed cell suspension of *Cl. welchii* was studied by coupling this reaction with dehydrogenation of glucose or ethyl alcohol and also with hydrogenase. The experimental results of the effects of various inhibitors on nitrate reducing activity of the organism, including those of addition of nitrate to culture medium, led to the conclusion that the nitrate reducing activity of *Cl. welchii* cells depended on the presence of nitrate reductase, participating in activation of nitrate molecule, distinct from dehydrogenases or hydro-

genase themselves. Some properties of the nitrate reductase of *Cl. welchii* was studied, especially in comparison with those of *E. coli*.

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REFERENCES

(*1*) Quastel, J. H., Stephenson, M., and Whetham, M. D., *Biochem. J.*, **19**, 304 (1925)
(*2*) Aubel, E., and Egami, F., *Bull. soc. chim. biol.*, **18**, 1542 (1936)
(*3*) Quastel, J. H., and Whetham, M. D., *Biochem. J.*, **18**, 519 (1924)
(*4*) Stickland, L. H., *Biochem. J.*, **25**, 98 (1931)
(*5*) Yamagata, S., *Acta Phytochim.* (*Japan*), **10**, 283 (1938) ; **11**, 145 (1939)
(*6*) Egami, F., and Sato, R., *J. Chem. Soc. Japan*, **68**, 39 (1947) ; 69, 20 (1948) ; **70**, 397 (1949)
(*7*) Joklik, W., *Australian J. Sci. Res.* B. **3**, 28 (1950)
(*8*) Sato, R., and Egami, F., *Bull. Chem. Soc. Japan.*, **22**, 137 (1949) ; Sato, R., and Niwa, M., *Bull. Chem. Soc. Japan* **25**, 202 (1952)
(*9*) Nicholas, D.J.D., Nason, A., and McElroy, Wm. D., *J. Biol. Chem.*, **207**, 341 (1954) ; Nicholas, D.J.D. and Nason, A., *J. Biol. Chem.*, **207**, 353 (1954)
(*10*) Woods, D. D., *Biochem. J.*, **32**, 2000 (1938)
(*11*) Katsura, T., Nemoto, M., Hirano, S., Nojima, T., and Egami, F., *J. Biochem.*, **39**, 38 (1952)
(*12*) Smith, W., Hale, J. H., and O'Callaghan, C. H., *J. Pathol. Bacteriol*, **65**, 229 (1953)
(*13*) Pappenheimer, Jr., A. M., and Shaskan, E., *J. Biol. Chem.*, **155**, 265 (1944)
(*14*) Waring, W. S., and Werkman, C. H., *Arch. Biochem.*, **1**, 303 (1942)
(*15*) Bard, R. C., and Gunsalus, I. C., *J. Bacteriol*, **59**, 387 (1950)
(*16*) Lardy, H. A., Respiratory enzymes, Burgess Publishing Co., Minneapolis (1949)
(*17*) Pappenheimer, Jr., A. M., *J. Biol. Chem.*, **167**, 251 (1947)
(*18*) Butlin, K. R. and Postgate, J. R., The 6th International Congress of Microbiology, Symposium microbial metabolism, p. 126 (1953)

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DISTRIBUTION OF RIBOFLAVIN NUCLEOTIDES IN RAT ORGANS INFLUENCED BY THE ADMINISTRATION OF FLAVIN COMPOUNDS

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Flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN), derivatives of free riboflavin, are indispensable substances in a living body, for they are prosthetic groups of yellow enzymes. Synthesis of FAD or FMN from free riboflavin is, therefore, important problem for the elucidation of a physiological meaning of riboflavin administered as a nutrient. Concerning this problem, FAD synthesizing enzyme was reported by Schrecker and Kornberg (*1*), and FMN synthesizing enzyme (flavokinase) was reported by Kearney and Englard (*2*). Already, synthesis of FAD or FMN by animal tissues have been reported by Rudy (*3*), Verzár *et al.* (*4, 5*), Klein and Kohn (*6*), Trufanov (*7, 8*), Makino *et al.* (*9*), and Yagi (*10, 11*). However, dynamic aspect or main route of the synthesis of FAD from free riboflavin in higher animals with reference to the destination of riboflavin administered to animal body has not been solved clearly.

To solve this problem, the changes in the distribution of riboflavin nucleotides in rat organs were measured after the administration of flavin compounds. The results are described in this paper.

METHOD AND MATERIALS

Measurement of nucleotides of riboflavin in rat organs was made by the microdetermination method using a filter paper chromatography (*12*). Flavin compound in homogenized tissue was extracted with water by heating the mixture at 80° for 15 minutes. Using an aliquot of this extract, the total quantity of flavin compounds was measured by the lumiflavin fluorescence method. From the rest of the extract, flavin compounds were extracted and separated on a filter paper, eluted and measured by the lumiflavin fluorescence method. From the total quantity of the flavin compounds and their rate, the absolute quantities were calculated.

Free riboflavin used in this experiments was extracted in a crystalline state from culture filtrate of *Eremothecium Ashbyii*, FAD was extracted from the pig liver and purified by a chromatopile (*13*), and FMN was obtained from FAD by hydrolysing it with trichloroacetic acid under a mild conditions (*14*).

For injection, these flavin compounds were dissolved in 0.9 per cent NaCl aqueous solution and their concentration was made up to 200 γ/ml., calculated as a free riboflavin.

EXPERIMENTAL

Distribution of Flavin Compounds in the Organs of Normal Rats—Male rats fed on a same food for over 10 days were starved for 24 hours. Soon after they were killed, the organs were excised and the quantity of each flavin compounds was determined. In the case of small organs, these from 2–5 rats were used for one measurement. The mathematical means of estimations of 5 experiments are shown in Table I. Each of 5 estimations gave simillar values. In the Table, the quantities of FAD and FMN were calculated as free riboflavin. From the results shown in Table I, it is clear that flavin compounds concentrate in the kidney, liver, and heart, and that FAD exists in a large quantity, while FMN is in a small amount and free riboflavin, in a minute amount. The ratio of the quantity of FMN to the total is small in case of the heart, and relatively large in the kidney, testicle, intestine, and brain.

TABLE I

Distribution of Flavin Compounds in Normal Organs of Rats

Organs	Total flavin compound	FAD		FMN		Free riboflavin	
Kidney	48.87 γ/g.	34.24 γ/g.	70.1 %	14.02 γ/g.	28.7%	0.61 γ/g.	1.2%
Liver	40.89	36.63	89.6	3.95	9.6	0.31	0.8
Heart	20.51	19.67	95.9	0.75	3.6	0.09	0.5
Stomach	7.70	6.78	88.0	0.78	10.1	0.14	1.9
Intestine	7.65	5.60	73.2	1.86	24.4	0.19	2.4
Spleen	6.72	6.05	90.0	0.54	8.1	0.13	1.9
Testicle	3.81	2.70	71.0	1.02	26.7	0.09	2.3
Brain	3.41	2.74	80.4	0.61	17.8	0.06	1.8
Muscle	3.01	2.75	91.5	0.22	7.2	0.04	1.3

Distribution of Flavin Compounds in the Organs of Rats when Free Riboflavin was Injected—Each of the male rats bred under the same conditions as those in the foregoing experiments was injected subcutaneously on their back with 300 γ of free riboflavin, and killed 30, 60 and 120 minutes after the injection. The quantity of flavin compounds found, is shown in Table II. Comparing Table II with Table I, it is noticed that marked change was observed only in the intestine, kidney, and liver, and the distribution of flavin compounds in the other organs remained unchanged.

TABLE II

Distribution of Flavin Compounds in Organs of Rats after Injection of Riboflavin

Organs	Time after injection	Total flavin compound	FAD		FMN		Free riboflavin	
Kidney	30*min.*	52.42 γ/g.	38.95γ/g.	74.3%	12.68γ/g.	24.2%	0.79γ/g.	1.5%
	60	55.35	43.01	77.7	11.51	20.8	0.83	1.5
	120	57.10	43.45	76.1	12.91	22.6	0.74	1.3
Liver	30	40.90	36.85	90.1	3.64	8.9	0.41	1.0
	60	47.82	43.61	91.2	3.64	7.6	0.57	1.2
	120	42.64	38.33	89.9	3.88	9.1	0.43	1.0
Heart	30	20.99	19.52	93.0	1.28	6.1	0.19	0.9
	60	20.85	19.70	94.5	1.04	5.0	0.11	0.5
	120	21.10	19.16	90.8	1.79	8.5	0.15	0.7
Stomach	30	6.10	5.40	88.5	0.56	9.2	0.14	2.3
	60	6.40	5.73	89.5	0.53	8.3	0.14	2.2
	120	7.27	6.47	89.0	0.57	7.8	0.23	3.2
Intestine	30	14.92	6.10	40.9	3.06	20.5	5.76	38.6
	60	25.00	5.40	21.6	9.10	36.4	10.50	42.0
	120	9.05	5.76	63.7	2.79	30.8	0.50	5.5
Spleen	30	5.67	5.19	91.6	0.38	6.6	0.10	1.8
	60	5.14	4.37	85.1	0.53	10.2	0.24	4.7
	120	6.53	5.77	88.3	0.56	8.6	0.20	3.1
Testicle	30	3.65	2.67	73.2	0.89	24.4	0.09	2.4
	60	4.00	2.91	72.8	1.00	24.9	0.09	2.3
	120	3.90	2.93	75.0	0.92	23.6	0.05	1.4
Brain	30	2.72	2.15	79.0	0.50	18.3	0.07	2.7
	60	3.37	2.72	80.7	0.59	17.5	0.06	1.8
	120	3.36	2.63	78.4	0.66	19.6	0.07	2.0
Muscle	30	2.99	2.68	89.7	0.26	8.5	0.05	1.8
	60	3.50	3.15	90.1	0.28	7.9	0.07	2.0
	120	2.70	2.43	90.0	0.23	8.5	0.04	1.5

In the case of the intestine, the quantities of FMN and free riboflavin increased and FAD remained unchanged, while in the case of the kidney and liver, only the quantity of FAD increased slightly.

When a section of the frozen, fresh small intestine was examined by a fluoromicroscope, a yellow green fluorescence was clearly observed in the mucous membrane but not in its muscular layer.

Distribution of Flavin Compounds in the Organs of Rats when FMN was Injected—Under the same conditions as in the above mentioned experiment, 300 γ of FMN (calculated as free riboflavin) was administered to each of the normal rats. The distribution of flavin compounds in the organs 60 minutes after the injection is shown in Table III. Also in this case, the quantity of flavin compounds increased only in the intestine, kidney, and liver. In the intestine, only FMN increased while in the kidney and liver, only FAD increased.

TABLE III

Distribution of Flavin Compounds in Organs of Rats after Injection of FMN

Organs	Total flavin compound	FAD		FMN		Free riboflavin	
Kidney	71.00γ/g.	56.73γ/g.	79.9%	13.63γ/g.	19.2%	0.64γ/g.	0.9%
Liver	52.33	48.14	92.0	3.87	7.4	0.32	0.6
Heart	21.46	20.62	96.1	0.73	3.4	0.11	0.5
Stomach	7.13	6.42	90.00	0.54	7.6	0.17	2.4
Intestine	9.55	5.23	54.8	3.82	40.0	0.50	5.2
Spleen	6.81	6.13	90.0	0.52	7.6	0.16	2.4
Testicle	4.12	3.24	78.6	0.76	18.5	0.12	2.9
Brain	4.15	3.11	75.0	0.91	22.0	0.13	3.0
Muscle	3.14	2.90	92.5	0.20	6.3	0.04	1.2

Distribution of Flavin Compounds in the Organs of Rats when FAD was Injected—Under the same conditions as in the above mentioned experiment, 300 γ of FAD (calculated as free riboflavin) was injected. The distribution of flavin compounds in organs 60 minutes after the injection is shown in Table IV.

TABLE IV

Distribution of Flavin Compounds in Organs of Rats after Injection of FAD

Organs	Total flavin compound	FAD		FMN		Free riboflavin	
Kidney	83.92γ/g.	70.74γ/g.	84.3%	12.42γ/g.	14.8%	0.76γ/g.	0.9%
Liver	61.70	57.69	93.5	3.70	6.0	0.31	0.5
Heart	24.91	23.94	96.1	0.85	3.4	0.12	0.5
Stomach	7.32	6.58	89.9	0.59	8.1	0.15	2.0
Intestine	10.37	6.88	66.3	2.95	28.5	0.54	5.2
Spleen	6.75	6.18	91.5	0.47	7.1	0.10	1.4
Testicle	3.80	2.85	75.2	0.85	22.3	0.10	2.5
Brain	3.60	2.99	83.1	0.55	15.2	0.06	1.7
Muscle	2.80	2.60	92.9	0.14	5.1	0.06	2.0

In this case, increase in the quantities of flavin compounds was observed only in the kidney, liver, heart, and intestine. FAD increased remarkably in the kidney and liver, and slightly in the heart. In the intestine, both FAD and FMN increased.

DISCUSSION

In normal rat organs, larger part of flavin compounds exists as FAD, and absolute quantity of the three forms of flavin compounds exist in almost a constant level.

The increase in the quantity of flavin compounds in several organs when riboflavin was administered, should be attributed to the concentration of administered riboflavin in these organs. The increase of FMN or FAD in these organs was naturally considered to be caused by the synthesis of these flavin compounds from riboflavin in these organs.

From the point of the above view, marked increase of flavin content in the intestine after the injection of riboflavin must have been caused by the concentration of injected riboflavin and the increase of FMN should be attributed to the phosphorylation of riboflavin in this organ. Slight increase of FAD in the liver and kidney suggests the possibility of either the synthesis of FAD in these organs or the concentration of FAD synthesized in other organs.

Definite increase of FAD in the liver and kidney, in the case of the administration of FMN, supports the possibility of the synthesis of FAD in these organs.

Increase of FAD in the liver, kidney, and heart in the case of the administration of FAD shows that these organs play some role in the storage of FAD.

Other organs which were not changed in flavin content by the administration of flavin compound might play no part in the synthesis or storage of FAD under normal conditions.

SUMMARY

1. A larger part of flavin compounds in rat organs existed in the form of FAD.

2. When riboflavin was administered to the back of a rat, FMN increased in the mucous membrane of the small intestine and FAD increased slightly in the liver and kidney.

3. When FMN was administered to a rat, FAD increased definitely in the liver and kidney.

4. When FAD was administered, FAD increased remarkably in the liver and kidney, and slightly in the heart.

From these results, it was considered that injected riboflavin was concentrated and phosphorylated in the mucous membrane of the small intestine, and then synthesized to FAD, chiefly in the liver and kidney.

REFERENCES

(*1*) Shrecker, A. W., and Kornberg, A., *J. Biol. Chem.*, **182**, 795 (1950)
(*2*) Kearney, E. B., and Englard, S., *J. Biol. Chem.*, **193**, 821 (1951)
(*3*) Rudy, H., *Naturwiss.*, **23**, 286 (1935)
(*4*) Hübner, H., and Verzár, F., *Helv. Chim Acta*, **21**, 1006 (1938)
(*5*) Pulver, R., and Verzár, F., *Enzymologia*, **6**, 333 (1939)
(*6*) Klein, J. R., and Kohn, H. I., *J. Biol. Chem.*, **136**, 177 (1940)
(*7*) Trufanov, A. V., *Biokhimiya*, **6**, 301 (1941)
(*8*) Trufanov, A. V., *Biokhimiya*, **7**, 188 (1942)
(*9*) Makino, K., Itoh, F., and Ohta, K., *J. Biochem.*, **37**, 459 (1950)
(*10*) Yagi, K., *Igaku to Seibutsugaku* (*Medicine and Biology*), **19**, 223 (1951) (in Japanese)
(*11*) Yagi, K., *Igaku to Seibutsugaku* (*Medicine and Biology*), **19**, 305 (1951) (in Japanese)
(*12*) Yagi, K., *J. Biochem.*, **38**, 161 (1951)
(*13*) Yagi, K., and Ishiguro, I., *J. Jap. Biochem. Soc.*, **22**, 162 (1950) (in Japanese)
(*14*) Bessey, O. A., Lowry, O. H., and Love, L. H., *J. Biol. Chem.*, **180**, 755 (1949)

The Journal of Biochemistry, Vol. 41, No. 6, 1954

BIOCHEMICAL STUDIES ON SULFATE-REDUCING BACTERIA

IV. THE CYTOCHROME SYSTEM OF SULFATE-REDUCING BACTERIA

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(Received for publication, September 9, 1954)

It is well known that sulfate-reducing bacteria are strict anaerobes, and use sulfate as hydrogen acceptor in place of oxygen. In the previous papers we reported that the sulfate-reducing bacteria isolated from paddy fields could utilize gaseous hydrogen, lactate, pyruvate and formate as hydrogen donors and reduce sulfate, sulfite, thiosulfate, tetrathionate and elemental sulfur to sulfide (*1*).

In this paper the presence of a cytochrome and another pigment in sulfate-reducing bacteria is reported. Cytochromes have generally been recognized as hydrogen carriers in respiratory enzyme systems for oxygen and have not been discovered in strictly anaerobic bacteria. But the strains of sulfate-reducing bacteria isolated in our laboratory were found to contain a cytochrome-type pigment. In the intact cell suspension the absorption bands of this pigment appear when reduced by gaseous hydrogen, formate, dithionite and other reducing agents, and disappear by oxidation with sulfate, sulfite, thiosulfate, hydroxylamine, air and other oxidants. Therefore it can be infererd that the pigment plays an important role in sulfate reduction by these sulfate-reducing bacteria.

Recently we were informed that Postgate had reported the presence of a cytochrome-type pigment from the same species of bacteria (*2*). It seems likely that we have been dealing with the same pigment as his.

MATERIALS AND METHODS

Strains and Culture—They are same as those described in the previous papers (*1*). The concentration of the intact cell suspension and the extract used are about 5 and 2 mg. N/ml., respectively.

Determination of Absorption Bands—For the purpose of the spectroscopic determination, the handspectroscope of Zeiss was used for cell suspensions and extracts, and Beckman spectrophotometer, Model DU, for the extracts. As the standard of wave length, cytochrome c prepared according to Keilin and Hartree was used which had a strong absorption band at 550 mμ in the reduced form (*3*).

EXPERIMENTS AND RESULTS

Observation with Intact Cell Suspension—Intact cell suspension had only a weak band at 630 mμ, but when a small amount of solid sodium dithionite was added, a strong band at 553 mμ and a weaker one at 520–527 mμ appeared besides that at 630 mμ. These bands except at 630 mμ disappeared on shaking with air and returned on standing in anaerobic state. From this observation two bands at 553 and 520–527 mμ may be presumably due to a cytochrome-type pigment.

When gaseous hydrogen, formate ($M/10$), or hydrogen sulfide ($M/10$) were used for reduction, the same bands of similar intensity were observed within 30 minutes. On the other hand, when lactate and pyruvate, which were used (likewise hydrogen and formate) as hydrogen donors in sulfate reduction, or succinate, glycerol and ethanol, which were not used, were added, only very faint bands at the same position appeared, on standing in anaerobic state.

When shaken in air, the bands disappeared immediately. This indicated the possibility that cytochrome oxidase might be present. But the test for cytochrome oxidase with Nadi reagent gave negative results. Further, these bacteria can not grow in aerobic condition, so these should be devoid of usual cytochrome oxidase observed in many aerobic organisms.

The Bands by Oxidation with Sulfate, Sulfite, Thiosulfate and Others—These observations were demonstrated in Thunberg tubes. 2 ml. of the intact cell suspension was put in the tube and 1 ml. of $M/10$ potassium sulfate, sodium sulfite or sodium thiosulfate in the hollow stopper. After the gas phase of each tube was replaced by gaseous hydrogen, it was incubated at 37°, until the strong absorption bands at 553 and 520–527 mμ appeared. Thereafter the tube was evacuated for 3 minutes by an aspirator, being tapped to release dissolved hydrogen, and the content of the hollow stopper was added into the tube. It was observed that the absorption bands at 553 and 527–520 mμ faded immediately and became very faint. The results are shown in Table I.

After the bands disappeared, the tube was flushed with hydrogen again and shaken. Then the bands re-appeared and faded gradually on standing. At the time hydrogen sulfide was developed, which could be demonstrated by the formation of yellow cadmium sulfide from cadmium carbonate added. These results indicate clearly that the cytochrome with bands at 553 and 520–527 mμ was oxidized by sulfate and reduced by hydrogen.

Observations with the Cell-free Extract—The centrifugate (40000×g, 20 minutes) of the bacterial extract was yellow in dilute state and dark brown in concentrated. At the observation with handspectroscope only an absorption band was observed at 630 mμ. But when a small amount of solid sodium dithionite was added to the ex-

Table I

Oxidation of the Cytochrome with Sulfate, Sulfite and Thiosulfate in Suspension of the Sulfate-reducing Bacteria

Acceptor added	Reaction time			
	0 min.	30 min.	60 min.	120 min.
Na_2SO_4	+++	++	+	+
Na_2SO_3	+++	++	+	+
$Na_2S_2O_3$	+++	++	+	+
None (water)	+++	+++	+++	+++

Cytochrome bands are; +, visible; ++, strong; +++, very strong.

tract, then absoption bands at 553 and 527–520 mμ, which could be observed with the intact cell suspension in reduced state, appeared. Therefore the cytochrome as well as the pigment with the band at 630 mμ was extracted in soluble state. In the precipitate of the centrigugation, only the weak bands of the cytochrome were perceptible after addition of dithionite. The absorption curves of the extract was determined with Beckman spectrophotometer. The results are indicated in Fig. 1. Absorption maxima were proved to be at 630 and 410 mμ in the oxidized state and at 630, 553, 523 and 419 mμ in the reduced, of which the bands at 553 and 523 mμ may be attributed to a cytochrome-type pigment. As the band at 630 mμ was not affected by reduction, it may be due to another pigment.

The properties of these pigments were tested. The pigment with the band at 630 mμ suffered no change with acid, but the cytochrome was easily precipitated at pH less than 5. When the precipitate was neutralized and reduced the absorption bands appeared again. The pigment with the band at 630 mμ was labile in alkaline solution and lost its band irreversibly. On the other hand, the cytochrome was quite stable in alkaline solution for several hours. On heating in neutral solution at 100° for 10 minutes, the cytochrome remained unchanged, but the other pigment lost its band at 630 mμ at 55° for 10 minutes.

By use of these properties, we tried to separate these pigments from each other. The extract was acidified with *N* HCl (below pH 4.0) and then centrifuged at 7000 ×*g* for 20 minutes. After the supernatant was poured away, the precipitate was dissolved with *M*/15 phosphate buffer, pH 7.0. The solution posessed bands only at 553 and 523 mμ and the supernatant only at 630 mμ.

The properties of the two pigments separated from each other were further tested. Both were not affected with cyanide or carbon monoxide. When a small amount of dithionite and 20 per cent pyridine were added, the band at 630 mμ disappeared at once and new bands were observed at 590 (weak and diffused) and 555–545 mμ (strong), and, on the other hand, the cytochrome showed somewhat stronger and broad bands

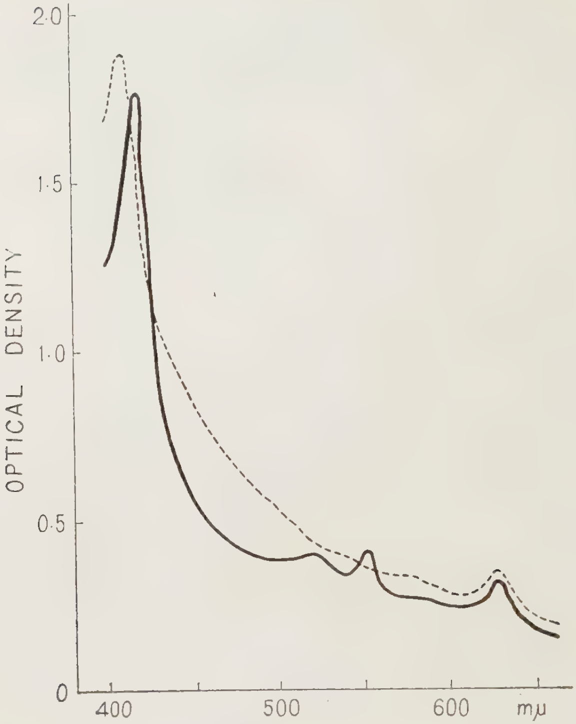

FIG. 1. Absorption spectra of the cell-free extract.
The extract (1 mg. N/ml.) was used for the measurement.
Light path: 1 cm.
- - - - - The extract.
——— The extract, reduced by the addition of a small amount of solid $Na_2S_2O_4$.

at the same position as in absence of pyridine.

In the cell-free extracts as well as in the intact cell suspension, the cytochrome was reduced by gaseous hydrogen, formate, sodium dithionite, hydrogen sulfide or cysteine. After heating at 100° for 10 minutes, the reduction was not demonstrated with hydrogen and formate, but easily with dithionite, hydrogen sulfide or cysteine, so the reduction with latters may take place non-enzymatically. Hydrogen and formate may reduce the pigment by means of hydrogenase and formic dehydrogenase, the presence of which were demonstrated previously (*1*). The reduced cytochrome is autoxidizable, for it is oxidized spontaneously even after heat treatment.

In order to estimate the oxidation-reduction potential of the cytochrome, we carried out experiments according to Ball's method (*4*). 2 ml. of the extract was placed in Thunberg tube and 1 ml. of oxidation-reduction dye solution in the hollow stopper. After the reduction of the cytochrome with gaseous hydrogen, the tube was evacuated for 3 minutes, and then the dye solution was poured in. Used dyes were methylene blue, nile blue, janus green, neutral red and methyl viologen. The added dye was partly reduced but the other part remained in oxidized form. The change of intensity of the cytochrome band at 533 mμ was observed (Table II).

TABLE II

Determination of rH_0 of the Cytochrome in Sulfate-reducing Bacteria

Dyes added	Methylene blue	Nile blue	Janus green	Neutral red	Methyl viologen
rH_0 of the dye	14.4	9.7	5.6	2.7	−0.9
Cytochrome band	−	−	+	+	+

The band disappeared instantly on adding methylene blue and nile blue, while the band remained even after 2 hours in the cases of janus green, neutral red and methyl viologen. Hence it was shown that the oxidation-reduction potential of this cytochrome should probably be between those of nile blue and janus green, *i.e.* between —0.14 and —0.25 volt at pH 7.0.

In cell-free extract, we could not observe oxidation of the cytochrome with sulfate, sulfite or thiosulfate, the last of which can be reduced to sulfide with hydrogen in the extract (*5*). On the other hand, if hydroxylamine (*M*/10, 1 ml.) was added instead of sulfate, *etc.*, the decrease of the intensity of the bands was apparently observed. It is quite natural to expect that this cytochrome is oxidized in the reduction of hydroxylamine, since the extract preparation seems to possess an ability for hydroxylamine reduction.

DISCUSSION

Up to date any cytochrome has not been found in strictly anaerobic bacteria. Keilin suggested that strict anaerobes could not synthesize prophyrin-ring in their cells (*6*). Contrary to his suggestion, cytochrome was recognized in sulfate-reducing bacteria which belong to strict anaerobes. As this cytochrome was reduced by hydrogen or formate, and oxidized by sulfate, sulfite and thiosulfate in cell suspension, it seems to play a important rôle in the sulfate reduction.

Egami and Sato reported on the relation between nitrate reduction and cytochrome system, and showed that cytochrome plays a role as intermidiary carrier in nitrate reduction as well as in aerobic respiration (*7*). But bacteria which they studied were facultative aerobes and had cytochrome originally in the cell. The presence of cytochrome in sulfate-reducing bacteria, not utilizing oxygen but sulfate, may presumably testify that cytochrome does not always concern with oxidation with oxygen alone.

The free energy changes in reduction of sulfate, *etc.*, with gaseous hydrogen are very much smaller than that of oxygen and nitrate, so the oxidation-reduction potentials is also low. The values of rH_0 calculated are 7.1 for sulfate—sulfide, 10.0 for sulfite—sulfide, 7.4 for thiosulfate—sulfide and 5.1 for sulfur—sulfide at pH 7.0. These values are comparable to that of oxido-reduction of organic metabolites. The fact that the cytochrome in the sulfate-reducing bacteria has rH_0 value between 9.3 and 5.7, indicates the possibility that the cytochrome may take part in the sulfate reduction as an intermediary carrier. In previous papers we suggested that sulfate reduction might take place by cooperation of sulfate reductase and hydrogenase or dehydrogenase connected with each other by some intermediary carriers (*1*). Whether the cytochrome is really the intermediate or not, is remained to be elucidated but the possibility can be considered.

The cell-free extract from the sulfate-reducing bacteria was found to possess and ability to reduce thiosulfate to sulfide and sulfite with gasseous hydrogen (*5*).

$$S_2O_3^{--} + H_2 = SO_3^{--} + H_2S.$$

The cytochrome bands, however, did not disappear by addition of thiosulfate. As the redox potential of the reduction of the thiosulfate is very much lower ($rH_0 = -0.1$) than that of the cytochrome, the possibility is left that a small portion of the cytochrome, reduced by hydrogen, is oxidized by thiosulfate and the reduction goes on, although the oxidized portion of the cytochrome was very small. In the case of addition of hydroxylamine, the potential of which is very much higher ($rH_0 = 39.6$), it is quite natural that the cytochrome is oxidized, as the hydroxylamine reductase is contained in the extract.

This cytochrome has absorption bands at 553 and 523 mμ in reduced state. In respect to the position and the heat stability of the bands, it resembles cytochrome c well, but is different from c as it is autoxidizable and has lower potential. Compared with cytochrome b_4, recently extracted from halophillic bacteria by Eagmi, Itahashi, Mori and Sato (*8*), it is very similar in the position of bands and instability of its reduced from, but its potential is higher than the cytochrome. Cytochromes b_2 (*9*) and c_1 (*10*) also differ at the position of bands of the hemochromogens. The cytochrome, therefore, seems to be a new type from the various properties, especially its low potential.

The other pigment in the sulfate-reducing bacteria has an absorption band at 630 mμ and forms hemochromogen with pyridine in reduced state. Its nature is not clear. There are several pigments, which have an absoption band near 630 mμ, namely catalase, horseradish peroxidase, myeloperoxidase, cytochrome c peroxidase (*11*) methemoglobin (*12*), coproporphyrin (*13*), and cytochrome a_2 (*14*). But cycochrome a_2, methemoglobin and peroxidases lose the band at 630 mμ by addition of dithionite; coproporphyrin does not form hemochromogen with pyridine, and hemochromogens of catalase and peroxidases have absorption bands at different positions from that of the pigment in the sulfate-reducing bacteria. So the pigment seems to be different from those, which have been found in other bacteria, but further investigation is necessary.

SUMMARY

Two strains of sulfate-reducing bacteria, isolated from paddy field, were found to possess a cytochrome-type pigment and another metal porphyrin pigment in spite of their strictly anaerobic characters. The former was reduced in living cells by gaseous hydrogen, formate and reducing agents, and oxidized by sulfate, sulfite and thiosulfate. Several properties of the two pigments in the crude extract were tested and separation of them was attempted.

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REFERENCES

(*1*) Ishimoto, M., Koyama, J., Omura, and T., Nagai, Y, *J. Biochem.* **41**, 537 (1954)
(*2*) Postgate, J., *Biochem. J.*, **56**, xi (1954)
(*3*) Keilin, D., and Hartree, E. F., *Proc. Roy. Soc.* (*London*), *B*, **122**, 289 (1937)
(*4*) Ball, E. G., *Biochem. Z.*, **295**, 262 (1938)

(*5*) Ishimoto, M., Koyama, J., and Nagai, Y., *J. Biochem.*, in press
(*6*) Keilin, D., *Biochem. J.*, **37**, xxi (1943)
(*7*) Sato, R., and Egami, F., *Bull. Chem, Soc. Japan* **22**, 137 (1949)
(*8*) Egami, F., Itahashi, M., Sato, R., and Mori, T., *J. Biochem.*, **40**, 527 (1953)
(*9*) Haas, E., Horecker, B. I., and Hogness, T. R., *J. Biol. Chem.*, **136**, 747 (1940)
Bach, J. S., Dixon, M., and Zerfas, L.G., *Biochem. J.*, **40**, 229 (1940)
Bach, J. S., Dixon, M., and Keilin, D., *Nature*, **149**, 21 (1942)
(*10*) Yakushiji, E., and Okunuki, K., *Proc. Imp. Acad.* (*Tokyo*), **16**, 299 (1940) : Okunuki, and K., Yakushiji, E., *Proc. Imp. Acad.* (*Tokyo*), **17**, 263 (1941)
(*11*) Theorell, H., The enzymes, Vol. II, p. 422 (1951)
(*12*) Yaoi, H., and Tamiya, H., *Proc. Imp. Acad.* (*Tokyo*), **4**, 436 (1928)
(*13*) Todd, C. M., *Biochem. J.*, **45**, 386 (1949)
(*14*) Tissieres, A., *Biochem. J.*, **50**, 279 (1950)

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METABOLISM OF HISTIDINE

V. FURTHER STUDIES ON FORMYL-L-GLUTAMIC DEFORMYLASE

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It was previously reported by us (*1*) that formyl-L-glutamic acid is an intermediate of L-histidine degradation by the microorganism employed. Independently Borek and Wealsch (*2*) found that formly-L-glutamic acid is decomposed rapidly by the extract of histidine-adapted *Pseudomonas fluorescens*.

Concurrently Borek and Waelsch (*2*) succeeded in isolating L-formamidino-glutaric acid from the reaction mixture of L-histidine and cat liver extract, and proposing the pathway of " urocanic acid, imidazolonpropionic acid, α-formamidinoglutaric acid, formylglutamic acid, and glutamic acid successively " as the metabolic pattern of L-histidine, which quite coincides with our opinion (*1*).

As to α-formamidinoglutaric acid, the authors (*3*) also confirmed the results of Borek and Waelsch by isolating the metabolite from the reaction mixture of urocanic acid and cat liver extract, and when bacterial enzyme was used to degrade urocanic acid, the accumulation of α-formamidinoglutaric acid was demonstrated paperchromatographically.

On the other hand Tabor *et al.* (*4*) isolated from the urine of folic acid deficient rats, later from the reaction mixture of urocanic acid and guinea pig liver extract, barium salt of a metabolite, one molecule of which is converted to each one molecule of ammonia, formic acid and glutamic acid by the extract of *Pseudomonas fluorescens*.

Furthermore, working on the bacterial enzyme system which catalyzes the conversion of the ' barium salt ' to glutamic acid, Tabor *et al.* (*5*) observed that when ' barium salt ' was incubated with H_2S-treated and aged extract of *Pseudomonas fluorescens*, glutamic acid was not obtainable but *N*-formyl-L-glutamic acid was isolated as the final reaction product accompanying the liberation of ammonia, and found that ferrous ion participates in the enzymatic deformylation of formyl-L-glutamic acid as a co-factor.

It was reported by us (*6*) that formyl-L-glutamic deformylase purified from bacterial origin (*Pseudomonas aeruginosa*) is a metal enzyme which requires cadmium, cobalt or ferrous ion. This result coincides with that of Tabor *et al.* so far as the nature of formyl-L-glutamic deformylase is a metal protein.

The experimental results concerning the adaptive nature of liver formyl-L-glutamic deformylase, its properties and substrate specificity of liver and bacterial enzyme, are presented in this paper.

MATERIALS AND METHODS

Formyl-L-glutamic acid, formylglycine, formyl-L-aspartic acid and formylanthranilic acid were prepared after du Vigneaud and Patterson's method (*7*). Formyl-DL-isoglutamine was isolated from the reaction mixture of urocanic acid and acetone powder of L-histidine-adapted cells.

Methods of determination of glutamic acid, formic acid and protein were the same as previously described (*6*).

The rates of decomposition of formylglycine, formyl-L-aspartic acid and formyl-DL-isoglutamine were measured by determining formic acid and ninhydrin-positive substances formed, the former by Grant's method (*8*), and the latter by Troll and Cannan's photometrical method (*9*). Formylanthranilic acid decomposed was determined spectrophotometrically by measuring the increase of optical density at 330 mμ.

Preparation of the Enzyme Extract—Guinea pigs weighing about 300 g. were used as experimental animals. The animal was injected intramuscularly 300 mg. of L-histidine monohydrochloride emulsified in rape oil, and sacrificed twenty hours later, then acetone powder of the fresh liver was prepared in the conventional method. The enzyme was extracted with 20 volumes (v/w) of $5 \times 10^{-2} M$ phosphate buffer (pH 5.6). This extract has no activities towards L-histidine and urocanic acid probably due to denaturation through acetone-treatment.

RESULTS AND DISCUSSION

Adaptive Increment of Formyl-L-glutamic Deformylase after Injection of L-Histidine

The above-prepared liver extracts of the histidine-injected guinea pigs were compared with those of the non-injected animals as to the enzymatic activity towards formyl-L-glutamic acid. As shown in Fig. 1, an average of specific activities of injected seven animals is 65 per cent more active than that of non-injected eight.

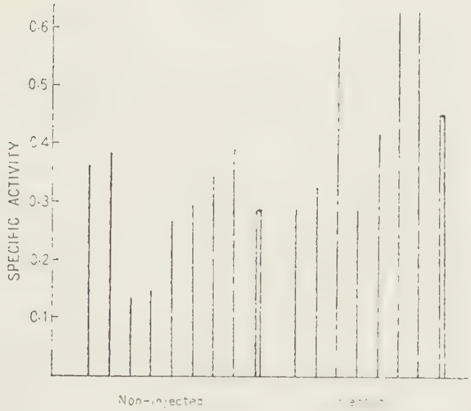


FIG. 1. Activities of the enzyme from L-histidine-injected and non-injected animals.

Experimental conditions: crude enzyme, 0.5 ml.; $2\times10^{-1}M$ phosphate buffer (pH 5.6), 0.5 ml.; $10^{-1}M$ formyl-L-glutamic acid, 0.2 ml.; final volume, 2.0 ml. with 0.8 ml. of distilled water; incubated at 37° for 1 hour.

Vertical line indicates specific activity, which represents micromoles of formyl-L-glutamic acid decomposed by 1 mg. protein at 37° for 1 hour. Blank lines indicate the averages of specific activities.

The result suggests that, in guinea pig liver, L-histidine is metabolized *via* formyl-L-glutamic acid; though it is reported by Borek and Waelsch (*2*) that in rat liver and rat kidney there is no enzymatic activity to decompose α-formamidinoglutaric acid.

Purification of the Enzyme and Its Properties

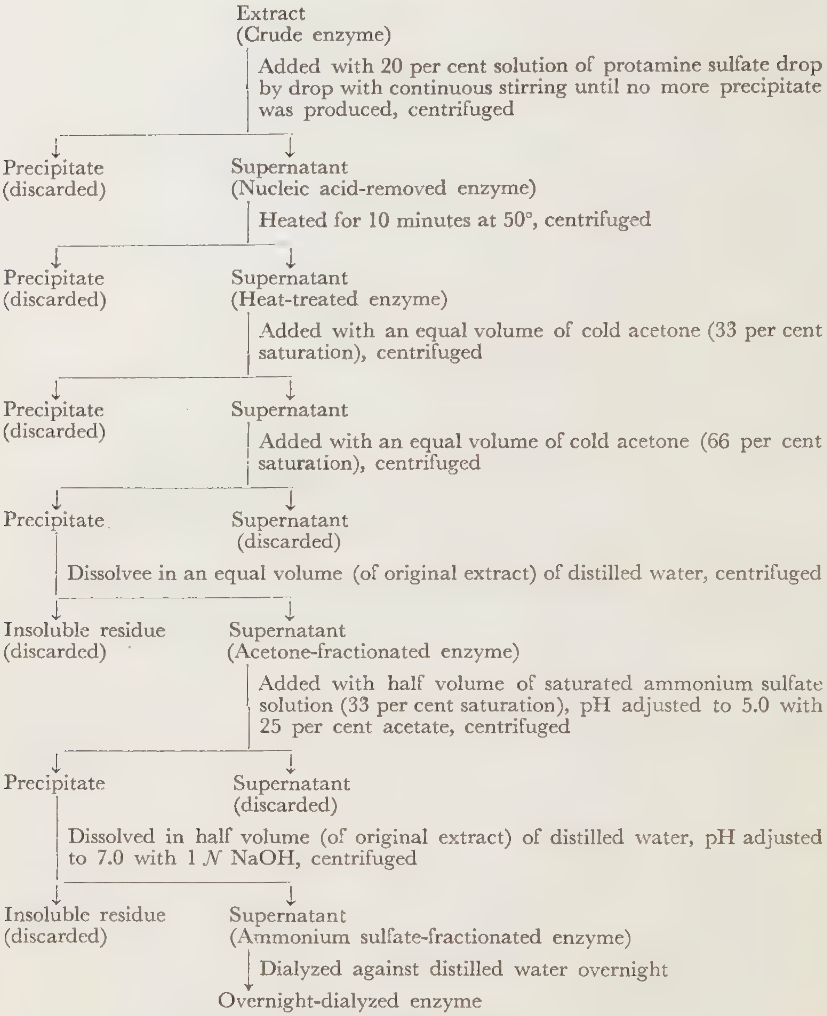
Purification of the Enzyme—The enzyme was purified as presented in Scheme 1. Protein content, specific activity and yield at each step of purification are summarized in Table I. When 2×10^{-3} *M* cobalt ion was added to the reaction mixture as described below, specific activity of the dialyzed enzyme was fifty-five times as active as the original crude enzyme.

pH Optimum of the Enzyme—pH optimum is found to be at 5.6 as shown in Fig. 2. The enzyme used in this experiment was the one dialyzed for 3 hours. Through the shorter period of dialysis, specific activity is not diminished to any extent.

Unlike the bacterial enzyme, it is observed that the liver enzyme is inhibited significantly by acetate buffer, *i.e.* 65 per cent in 5×10^{-3} *M* concentration.

Effects of Inhibitors—$2\times10^{-3}M$ α,α′-dipyridyl and ethylenediamine tetraacetate (EDTA) inhibit the enzymatic reaction 100.0 and 68.0 per cent respectively. Unlike

SCHEME I



Extract
(Crude enzyme)
Added with 20 per cent solution of protamine sulfate drop by drop with continuous stirring until no more precipitate was produced, centrifuged

Precipitate (discarded)
Supernatant (Nucleic acid-removed enzyme)
Heated for 10 minutes at 50°, centrifuged

Precipitate (discarded)
Supernatant (Heat-treated enzyme)
Added with an equal volume of cold acetone (33 per cent saturation), centrifuged

Precipitate (discarded)
Supernatant
Added with an equal volume of cold acetone (66 per cent saturation), centrifuged

Precipitate
Supernatant (discarded)
Dissolvee in an equal volume (of original extract) of distilled water, centrifuged

Insoluble residue (discarded)
Supernatant (Acetone-fractionated enzyme)
Added with half volume of saturated ammonium sulfate solution (33 per cent saturation), pH adjusted to 5.0 with 25 per cent acetate, centrifuged

Precipitate
Supernatant (discarded)
Dissolved in half volume (of original extract) of distilled water, pH adjusted to 7.0 with 1 *N* NaOH, centrifuged

Insoluble residue (discarded)
Supernatant (Ammonium sulfate-fractionated enzyme)
Dialyzed against distilled water overnight

Overnight-dialyzed enzyme

the bacterial enzyme, the liver enzyme is not inhibited by $2\times10^{-3}M$ 8-hydroxyquinoline. *p*-Chloromercuribenzoate shows a perfect inhibitory effect in $10^{-3}M$ concentration, and even in the presence of $10^{-2}M$ glutathione the enzyme activity cannot be preserved from this complete inhibition. The experimental results are presented in Table II.

TABLE I

	Volume	Total protein	Specific* activity	Yield
	ml.	*mg.*		%
Crude enzyme	38.0	958.0	0.63 (Co^{++}) 0.75	100.0 (Co^{++}) 119.0
Nucleic acid-removed enzyme	40.0	822.0	0.76	103.0
Heat-treated enzyme	37.0	518.0	0.97	83.4
Acetone-fractionated enzyme	40.0	213.0	1.85	65.2
Ammonium sulfate-fractionated enzyme	20.0	10.8	13.70 (Co^{++}) 37.20	24.5 $Co^{++})$ 66.5
Overnight-dialyzed enzyme	22.0	7.8	10.00 (Co^{++}) 41.60	12.9 (Co^{++}) 53.8

0.5 ml. of the enzyme solution at each step of purification was incubated with 10^{-2} *M* formyl-L-glutamic acid in $5\times10^{-2}M$ phosphate buffer (pH 5.6) at 37° for 1 hour. Total volume was 2.0 ml.

* Specific activity represents micromoles of formyl-L-glutamic acid decomposed by 1 mg. of protein at 37° for 1 hour.

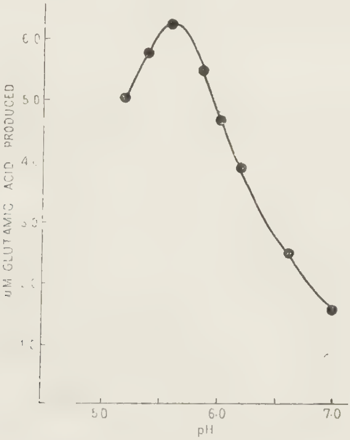


FIG. 2. pH optimum of the enzyme.

Experimental conditions: 3 hours-dialyzed enzyme, 0.5 ml. containing 234 γ protein; $2\times10^{-1}M$ phosphate buffer (pH 5.6), 0.5 ml.; $10^{-1}M$ formyl-L-glutamic acid, 0.2 ml.; final volume, 2.0 ml. with 0.8 ml. of distilled water; incubated at 37° for 2 hours.

TABLE II

Inhibitor*	Reaction product		Inhibition*** ratio
	Glutamic acid	Formic acid	
None	4.86 μM	4.76 μM	%
8-Hydroxy-quinoline**	4.86	4.80	0.0
α, α′-Dipyridyl	0.00	0.00	100.0
EDTA	1.55	1.48	68.0
p-Chloromercuribenzoate	0.00	0.00	100.0
,, + Glutathione	0.00	0.00	100.0

Experimental conditions: dialyzed enzyme, 0.5 ml. containing 177 γ protein; $2\times10^{-1}M$ phosphate buffer (pH 5.6), 0.5 ml.; $10^{-1}M$ formyl-L-glutamic acid, 0.2 ml.; inhibitors, *i.e.* $10^{-1}M$ 8-hydroxyquinoline in ethanol, $10^{-2}M$ α,α′-dipyridyl, $10^{-2}M$ EDTA and $10^{-2}M$ *p*-chloromercuribenzoate, 0.04 ml., 0.4 ml., 0.4 ml., 0.4 ml. and 0.4 ml. respectively; $10^{-1}M$ glutathione, 0.4 ml.; final volume, 2.0 ml. with adequate amounts of distilled water; incubated at 37° for 2 hours.

* Inhibitors used were without action on the enzymatic activity of glutamic decarboxylase.

** Two per cent ethanol was not inhibitory on the enzymatic activity of formyl-L-glutamic deformylase.

*** Inhibition ratios were calculated from the amounts of glutamic acid produced.

TABLE III

Metal ions	Concentration optimum	Glutamic acid produced	Specific activity	Activation ratio
None	*M*	2.45 μM	13.8	1.00
Co^{++}	2×10^{-3}	10.20	57.6	4.16
Ni^{++}	10^{-3}	9.37	53.0	3.78
Zn^{++}	5×10^{-4}	7.85	44.3	3.20
Cd^{++}	2.5×10^{-4}	7.75	43.8	3.16
Fe^{++}+V.C	10^{-3}	5.82	32.9	2.37

Experimental conditions: dialyzed enzyme, 0.5 ml. containing 177 γ protein; $2\times10^{-1}M$ phosphate buffer (pH 5.6), 0.5 ml.; $10^{-1}M$ formyl-L-glutamic acid, 0.2 ml.; salt solutions, varying in concentrations; $2\times10^{-2}M$ V. C, 0.2 ml.; final volume, 2.0 ml. with adequate amounts of distilled water; incubated at 37° for 1 hour.

These results suggest the participation of a certain metal ion and the necessity of -SH group for the activity of this enzyme.

Activating Effects of Cobalt, Nickel, Zinc, Cadmium and Ferrous Ions—Effects of various metal ions were examined with overnight-dialyzed enzyme. No effects were observed with Li^+, Mg^{++}, Ca^{++}, Sr^{++}, Mn^{++} and Al^{+++}, inhibitory effects with Ag^+, Hg^{++}, Cu^{++}, Pb^{++}, Cr^{++}, Ba^{++} and Fe^{+++}, and it was found that Co^{++}, Ni^{++}, Zn^{++}, Cd^{++} and Fe^{++}+V. C remarkably activate the enzyme action. Maximum-activating concentrations of these metal ions together with the degree of the enhancement of the activities are presented in Table III. Fe^{++}, if not added with vitamin C (V. C), inhibits 40 per cent or thereabouts: probably because of oxidation of Fe^{++} to Fe^{+++}.

When the overnight-dialyzed enzyme was dialyzed for further 24 hours, the enzyme activity is lost completely. This inactivated enzyme regains its activity by the addition of the above-described metal ions. Cd^{++} gives the best reactivating effect and the other metal ions, Co^{++}, Ni^{++}, Zn^{++} and Fe^{++}+V.C, are much less effective than Cd^{++}, but as shown in Table IV, the maximal recovery even with Cd^{++} remains only one-third or thereabouts as compared with the restored activity of the overnight-dialyzed enzyme presented in Table III. Maybe, through further dialysis, partial denaturation of the enzyme protein takes place.

TABLE IV

Concentration of Cd^{++}	Glutamic acid produced	Specific activity
M 5×10^{-4}	μM 2.60	8.1
2.5×10^{-4}	5.42	16.9
1.25×10^{-4}	2.36	7.4
6.25×10^{-5}	1.50	4.7

Experimental conditions: further dialyzed inactivated enzyme, 0.5 ml. containing 160 γ protein; $2\times10^{-1}M$ phosphate buffer (pH 5.6), 0.5 ml.; $10^{-1}M$ formyl-L-glutamic acid, 0.2 ml.; $10^{-2}M$ cobalt sulfate, varying in concentrations; final volume, 2.0 ml. with adequate amounts of distilled water; incubated at 37° for 1 hour.

Substrate Specificity of the Enzyme

We examined to find out what differences of enzymatic activities exsist between bacterial and liver enzyme, towards the various *N*-formyl-compounds, that is, formylglycine, formyl-L-aspartic acid, formyl-DL-isoglutamine and formylanthranilic acid.

Liver enzyme, both crude and purified, attacks formylglycine and formyl-L-aspartic acid, the former at a considerably rapid rate, the latter much slower, as shown in Table V. Crude bacterial enzyme decomposes formly-L-aspartic acid solely at a very slow rate. Non-adapted cells of the microorganism employed has, so called constitutively, a considerable activity to decompose formyl-L-glutamic acid. When adapted to L-histidine, the activity towards formyl-L-glutamic acid increases about three times. Proportionally to this increment, the activity towards formyl-L-aspartic acid equally increases, but this activity is removed through purification as shown in Table V.

TABLE V

Enzyme \ Substrate		*N*-Formyl-compounds decomposed (%)				
		Formyl-L-glutamic acid	Formyl-glycine	Formyl-L-aspartic acid	Formyl-DL-isoglutamine	Formyl-anthranilic acid
Liver enzyme	(1) Crude (nucleic acid-removed) enzyme	64.8	41.0	3.6	0.0	0.0
	(2) Purified enzyme	47.0	21.4	2.3	0.0	0.0
Bacterial enzyme	(3) Acetone powder of normal cells	30.0	0.0	3.2	0.0	0.0
	(4) Acetone powder of L-histidine-adapted cells	88.0	0.0	8.8	0.0	0.0
	(5) Purified enzyme	65.0	0.0	0.0	0.0	0.0

Experimental conditions of (1) and (2): enzyme solution, 0.5 ml. [(1), crude enzyme containing 10.5 mg. protein; (2), purified enzyme containing 177 γ protein]; $2\times10^{-1}M$ phosphate buffer (pH 5.6), 0.5 ml.; $10^{-1}M$ substrates, 0.2 ml.; $2\times10^{-2}M$ cobalt chloride, 0.2 ml.; final volume, 2.0 ml. with 0.6 ml. distilled water; incubation at 37° for 1 hours.

(3) and (4): suspension of acetone powder in $5\times10^{-2}M$ acetate buffer pH 6.2, 1.0 ml. containing 20 mg. acetone powder [(3), non-adapted; (4), L-histidine-adapted]; $2\times10^{-1}M$ acetate buffer (pH 6.2), 0.25 ml.; $10^{-1}M$ substrates, 0.2 ml.; final volume, 2.0 ml. with 0.55 ml. distilled water; incubated at 37° for 1 hour.

(5): purified enzyme, 0.5 ml. containing 230 γ protein; $2\times10^{-1}M$ acetate buffer (pH 6.2), 0.5 ml.; $10^{-1}M$ substrates, 0.2 ml.; $10^{-2}M$ cadmium sulfate, 0.2 ml.; final volume, 2.0 ml. with 0.6 ml. distilled water; incubated at 37° for 1 hour.

Knox and Mehler (*10*) prepared formylase from rabbit and rat liver, and lately Jakoby (*11*) reported about kynurenine formamidase from *Neurospora*: these two enzymes closely resemble each other in respect of substrate specificity, but differ from our formyl-L-glutamic deformylase as shown in Table VI.

TABLE VI

Enzyme / Substrate	Formylase from liver (Knox, Mehler)	Kynurenine-formanidase from Neurospora (Jakoby)	Formyl-L-glutamic deformylase	
			Liver	Bacteria
Formyl-L-glutamic acid		−	+	+
Formylanthranilic acid	+	+	−	−
Formylglycine	−	−	+	−
Formyl-L-aspartic acid	−	−	+	−

These results suggest that *N*-formyl-compounds formed as an intermediate of amino acid metabolism are by no means decomposed by a single enzyme, non-specific formylase.

Differences between Bacterial and Liver Purified Enzyme

The differences between bacterial and liver enzyme are summarized in Table VII.

TABLE VII

	Bacterial purified enzyme	Liver purified enzyme
Dialysis	Activity not decreased	Activity decreased
pH optimum	6.2	5.6
5×10^{-2} *M* Acetate buffer	No inhibition	Significant inhibition
2×10^{-3} *M* 8-Hydroxyquinoline	Significant inhibition	No inhibition
10^{-3} *M p*-Chloromercuribenzoate	Partial inhibition	Complete inhibition
Zn^{++}, Ni^{++}	No activation	Significant activation
Formylglycine, Formyl-L-aspartic acid	Not decomposed	Decomposed

SUMMARY

1. When L-histidine was injected to guinea pig before being sacrificed, the activity of liver formyl-L-glutamic deformylase is increased adaptively.

2. Formyl-L-glutamic deformylase was extracted from acetone powder of guinea pig liver and purified about fifty-five folds.

3. pH optimum of the enzyme action lied at 5.6.

4. The enzyme was irreversibly inactivated by *p*-chloromercuribenzoate.

5. The enzyme action was inhibited by various metal-binders, and the dialyzed enzyme was significantly activated with Co^{++}, Ni^{++}, Zn^{++}, Cd^{++} or Fe^{++}+V. C.

6. Liver enzyme, either crude or purified, had the activities towards formylglycine and formyl-L-aspartic acid. Bacterial enzyme contained a slight activity towards formly-L-aspartic acid and this activity was removed through purification.

7. Differences of properties between bacterial and liver enzyme were summarized.

REFERENCES

(*1*) Suda, M., Nakaya, A., Hara, M., Kato, A., and Ikenaka, T., *Med. J. Osaka Univ.*, **4**, 107 (1953)
(*2*) Borek, B. A., and Waelsch, H., *J. Biol. Chem.*, **205**, 459 (1953)
(*3*) Kato, A., and Nakaya, A., Annual Meeting of the Society of Japanese Biochemists, Sendai (1954)
(*4*) Tabor, H., Silverman, M., Mehler, A. H., Daft, F. S., and Bauer, H., *J. Am. Chem. Soc.*, **75**, 756 (1953)
(*5*) Tabor, H., and Mehler, A. H., *Federation Proc.*, **18**, 409 (1954)
(*6*) Kato, A., Nakaya, A., Watanabe, M., and Suda, M., *Med. J. Osaka Univ.* **5**, 359 (1954)
(*7*) du Vigneaud, V., and Patterson, W. I., *J. Biol. Chem.*, **109**, 97 (1935)
(*8*) Grant, W.M., *Ind. Eng. Chem.*, **18**, 729 (1946)
(*9*) Troll, W., and Cannan, K., *J. Biol. Chem.*, **200**, 803 (1953)
(*10*) Knox, W. E., and Nehler, A. H., *J. Biol. Chem.*, **187**, 431 (1950)
(*11*) Jakoby, W. B., *J. Biol. Chem.*, **207**, 657 (1954)

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SYNTHESIS OF GLUCURONIDES BY TISSUE SLICES

II. SYNTHESIS AND PROPERTIES OF α-D-GLUCURONIC ACID-1-PHOSPHATE

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In the previous paper (*1*) we have reported that the glucuronide synthesis by liver slices is markedly augmented by the addition of Cori's ester. The finding is in good agreement with the results obtained by isotopic tracer studies performed in North America; thus Mosbach and King (*2*), Eisenberg and Gurin (*3*), and Douglas and King (*4*) showed that in the administration of C^{14} labeled glucose to the animal, C^{14} labeled glucruronic acid was formed, and moreover demonstrated that the carbon atom skeleton of administered glucose was directly used for the formation of glucuronic acid—for instance, from 1-C^{14}-glucose the glucuronic acid in which C^{14} was concentrated at 1-position was formed. But in those cases, only a few per cent of C^{14} of the administered glucose was incorporated into glucuronic acid, so that it may as well be possible that glucose is not the direct precursor of glucuronic acid.

The previous results obtained by our slice method and those isotopic studies by American authors suggest that Cori's ester may be a key substance in glucuronide synthesis; and if this be true, the next metabolite must be glucuronic acid-1-phosphate. Of the phosphoric acid derivative, the β-form was previously synthesized by Touster and Reynolds (*5*), and was proved to be inactive in glucuronide synthesis; α-glucuronic acid-1-phosphate has been synthesized and partially purified by us, and the present paper deals with the synthesizing method, general properties and its effects on the conjugation of anthranilic acid as glucuronide by rat liver slices.

EXPERIMENTALS AND RESULTS

Synthesis of α-D-Glucuronic Acid-1-phosphate—This substance has succesfully been synthesized by the oxidation of Cori's ester with hydrogen peroxide. Cori's ester used was prepared from starch by the method described by Hanes (*6*). Hydrogen peroxide should be made first quantitatively free from sulufuric acid (being added as the stabilizer) by the addition of barium hydroxide.

5.0 g. of Cori's ester was dissolved in 250 ml. of water and 10 ml. of 12 per cent hydrogen peroxide was added. The oxidation was continued for 6 days at room temperature and supplementary 5 ml. of the hydrogen peroxide solution was added daily once. On the 7th day pH of the mixture was adjusted to 7.0 by potassium hydroxide and the solution was diluted up to 600 ml. The remaining peroxide was removed with platinum asbestos—the process was carried out in a refrigerator overnight. After the platinum asbestos was removed by filtration, the solution was concentrated *in vacuo* to 100 ml. and its pH was adjusted to 8.0 by potassium hydroxide, and finally 25 per cent barium acetate solution was added in the ratio of 1 ml. to 1 mg. of total phosphorus in the reaction mixture. After standing for 30 minutes at room temperature, the solution was centrifuged. To the supernatant solution 400 ml. of 0.23 N barium hydroxide solution was added by vigorous stirring and the mixture was allowed to stand overnight in the refrigerator. The white precipitate thus formed was collected by centrifugation, washed with water, ethanol and ether, and lastly dried *in vacuo*—the yield of crude barium salt being about 1 g.

The purification of crude salt was conducted as follows: The material was extracted several times with warm water. The combined extracts were concentrated *in vacuo* to an appropriate volume and the salt was precipitated by adding fivefold 95 per cent ethanol. The mixture was allowed to stand overnight in the refrigerator, the precipitate thus formed was collected by centrifugation and dried in vacuum over calcium chloride. The yield of the purified barium salt was about 0.4 g.

Synthesis of β-D-Glucuronic acid-1-phosphate—To compare the properties of α- and β-isomers, the β-form was prepared by the method described by Touster and Reynolds (*5*). The crude barium salt obtained was purified either by the similar way used for the α-isomer or by its transformation into brucine salt.

Properties of α- and β-D-Glucuronic acid-1-phosphates—These properties are summarized in Tables I and II.

The barium contents in both α- and β-derivatives are somewhat higher than the

TABLE I

Qualitative Properties

Property	α-Form	β-Form
Naphthoresorcinol reaction	+	+
Phloroglucinol reaction	+	+
Inorganic phosphorus	trace	trace
Organic phosphorus	+	+
Reduction*	—	—
Reduction after hydroylsis**	+	+

* Benedict's test.

** Treatment with N sulufuric acid at 100° for 15 minutes.

TABLE II

Elemental Analyses and Specific Rotations of α- and β-Isomers

Isomers	C	H	Ba	Total P	H_2O†	$[\alpha]_D^{23}$*
α	12.88	2.94	42.15	6.01	—	+14.0
β	12.93	2.80	41.98	5.90	12.36	− 2.6
Theoretical ($C_6H_9O_6PO_4\cdot 1\frac{1}{2}Ba\cdot 4H_2O$)	13.09	3.09	37.63	5.63	13.02	—

† Loss of weight after drying *in vacuo* at 80° for 5.5 hours.

* The barium salt was dissolved in 0.15 *N* hydrochloric acid. c=1.0 per cent, l=1.0 dm.

TABLE III

Effects of α- and β-D-Glucuronic Acid-1-phosphates on the Conjugation of Anthrnilic Acid by Rat Liver Slices

Concentration of glucuronic acid-1-phosphate	Conjugated anthranilic acid	Activation (+) or inhibition (−)
	α-Isomer from barium slat	
M	γ	%
0	9.9	—
2×10^{-2}	5.1	−48.4
1×10^{-2}	9.3	− 6.6
1×10^{-3}	13.9	+39.7
1×10^{-4}	17.9	+81.8
	β-Isomer from barium salt	
M	γ	%
0	14.0	—
2×10^{-2}	6.4	−54.3
1×10^{-2}	10.8	−22.6
1×10^{-3}	12.4	−11.4
1×10^{-4}	13.4	− 4.3
	β-Isomer from brucine salt	
M	γ	%
0	9.2	—
1×10^{-2}	5.2	−43.4
1×10^{-3}	6.0	−34.7
1×10^{-4}	9.0	− 2.0

theoretical value. It may minly be due to a contamination of barium carbonate. The purity of samples seemed to be around 90 per cent.

Effects of the α- and β-Derivatives on the Glucuronide Synthesis—The assay of glucuronide synthesis was conducted by the method previously reported by us (*7*). The derivatives were freed quantitatively from barium by addition of dilute sulufuric acid, before these were placed into the reaction mixture.

In case of β-derivative, free acid was prepared from brucine salt as well as from barium salt by the extraction of its alkaline solution with chloroform.

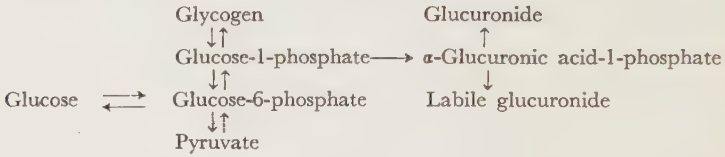
All these operations were performed at low temperature to avoid hydrolytic removal of phosphoric acid rest.

The effects of these derivatives on the conjugation of anthranilic acid as glucuronide by rat liver slices are summarized in Table III.

As shown in the data α-derivative, even though acted inhibitory at higher concentration, augmented the conjugation at lower concentration ($10^{-3}\sim10^{-4}\,M$) more powerfully than Cori's ester did at its optimal concentration ($2\times10^{-2}\,M$) (*1*). β-Derivatives, on the other hand, did not stimulate anthranilic acid glucuronide formation at all the concentrations tested.

DISCUSSION

From the data described above, glucuronide synthesis may proceed according to the following scheme.



Of course, the scheme may only be valid as a general description of the process, and many complicated reactions must undoubtedly be involved herein. Recently, Dutton and Storey (*8*) have found that a substance which is likely to be uridine-diphosphoric acid-glucuronic acid, behaves as a cofactor in the conjugation of *o*-aminophenol. The details of their experiment are still obscure, but it may not be wrong to regard that the substance isolated by the English authors is closely allied to our α-glucuronic acid-1-phosphate.

SUMMARY

1. α-D-Glucuronic acid-1-phosphate was synthesized, which showed a high activity in the present glucuronide synthesis.

2. β-D-Glucuronic acid-1-phosphate was proved to be inactive as previously reported by other authors.

The authors wish to thank Prof. Mabuti for his continued interest in this problem.

REFERENCES

(*1*) Shirai, Y., and Ohkubo, T., *J. Biochem.*, **41**, 341 (1954)
(*2*) Mosbach, E. H., and King, C. G., *J. Biol. Chem.*, **185**, 491 (1950)
(*3*) Eisenberg, F. J., and Gurin, S., *J. Biol. Chem.*, **195**, 317 (1952)
(*4*) Douglas, J. F., and King, C. G., *J. Biol. Chem.*, **202**, 865 (1953)
(*5*) Touster, O., and Reynolds, V. H., *J. Biol. Chem.*, **197**, 863 (1952)
(*6*) Hanes, C. S., *Proc. Roy. Soc.*, (*London*) *B*, **129**, 175 (1940)
(*7*) Shirai, Y., and Ohkubo, T., *J. Biochem.*, **41**, 337 (1954)
(*8*) Dutton, G. J., and Storey, I.D.E., *Biochem. J.*, **53**, xxxvii (1953)

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ISOTOPIC STUDIES ON THE METABOLISM OF PHOSPHATE COMPOUNDS IN THE CELLULAR FRACTIONS OF RAT LIVER

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It has been well known for many years that some kinds of components in the cellular structure play an important rôle in the metablism. In recent years, by the efforts of Claude, Hoogeboom, Schneider and their colleagues (*1–4*), it has become possible to isolate, by means of differential centrifugation, at least four cellular fractions (nuclei, mitochondria, microsomes, and cell fluid) from animal tissues. Since then the localization of enzymes in such celluallar components has been studied by many workers, and, as a result, it is now established that the mitochondria is the major site of the oxidation and coupled phosphorylation of the intermediates of Krebs tricarboxylic acid cycle (*5–11*) and of the oxidation of fatty acids (*12*) and that the supernatant fraction contains glycolytic enzymes and the nuclear fraction, a large part of arginase (*13*).

However, there have been only few reports concerning the metabolic activities of the celullar components in vivo. Experiments with isotopes represent a feasible method of approach to secure such informations but there have been few publications, in which the renewal rate of nucleic acid or the rate of incorporation of amino acids into protein was compared in each cellular component (*14–17*). To clarify in detail the rôle of cellular components in the phosphorus metabolism, the following experiments were undertaken by the present authors. The renewal rates of acid-soluble and insoluble phosphate compounds in isolated cellular components of rat liver were compared at various time intervals after the injection of isotopic inorganic P^{32}, and a special attention was paid to the initial short period after the administration of the isotope.

EXPERIMENTAL

Rats weighing 150–200 g. were used in the experiments. They received a nutritionally complete synthetic stock ration *ad libitum*. For the turnover experiments, rats were fasted for about 17 hours and intraperitoneally injected with $P^{32}O_4$ as 2 ml. carrier-free sodium salt (pH 7.4). The dose of P^{32} was about 30 microcuries (μc) per 100 g. of body weight. About 5 minutes before the end of the experiment, the peritoneal and thoracic cavities were opened, and 1 ml. of blood was taken as quickly as possible from the heart. The liver was removed after being perfused for 5 minutes with about 100 ml. of ice-cold 0.9 per cent NaCl solution. The time necessary to perfuse liver was 5 minutes. The following manipulation was perfomed in a cold room (0 to 2°). The liver was passed through a tissue masher. The liver pulp was then weighed and homogenized in nine volumes of cold isotonic sucrose solution using Potter and Elvehjem glass homogenizer (*18*). The fractionation of particulate components of the liver cells was carried out by the Schneider centrifugal fractionation procedure using an isotonic sucrose solution (*4*). The following cell components were separated: the nuclear fraction (N-fr), the mitochondrial fraction (Mt-fr), and the supernatant fraction including microsomes (S-fr). Each fraction was diluted with water to a definite volume and an aliquot was taken, deproteinized with trichloroacetic acid (final concentration 7 per cent), and then centrifuged. The precipitate was washed with 5 per cent trichloroacetic acid, and the supernatant and washings were combined for the analysis of acid-soluble phosphate compounds. The residue was used to prepare acid-insoluble phosphate compounds.

A suitable portion of each trichloroacetic acid extract was taken and P^{31} and P^{32} concentrations of inorganic P, $\Delta 7$P, and organic P other than $\Delta 7$P of each cellular fraction were determined. Then the separation of the ATP-ADP fraction and the inorganic P fractions of whole homogenate and Mt-fr was carried out by the method reported in the previous paper (*19*), and P^{31} and P^{32} concentrations of the labile P in the ATP-ADP fraction and inorganic P in the inorganic P fraction were determined. The separation of the ATP-ADP fraction of N-fr and S-fr was not successful, because the concentration of ATP-ADP in N-fr was very low, and the high concentration of sucrose of S-fr prevented the precipitation of ATP and ADP as their Ba salts.

For the fractionation of the acid-insoluble phosphate compounds, the modified methods (*19*) of Schmidt-Thanhauser (*21*) and of Schneider (*20*) were employed, and P^{31} and P^{32} concentrations of lipids, ribonucleic acid (RNA), and desoxyribonucleic acid (DNA) in each cellular fraction were determined.

A suitable portion of each of the above fractions was taken for the determination of P by the method of Fiske-Subbarow (*22*).

For the measurement of the radioactivity of inorganic P^{32}, isobutanol method of Enner and Rosenberg (*23*) was employed, and $\Delta 7P^{32}$ of the acid-soluble fraction and ATP-ADP fraction was measured as the inorganic P^{32} after ten minutes' hydrolysis in 1 *N* HCl and precipitation as $MgNH_4PO_4$. A minitue amount of inorganic P^{32} contaminated in ATP-ADP fraction was removed by the isobutanol method and this value was corrected for the calculation of labile P^{32} of ATP-ADP. For the measurement of total acid-soluble P^{32}-phosphate compounds, an aliquot of each soluble fraction was taken up in a small glass dish with a filter paper, dried completely by infrared rays, and the radioactivity was measured.

The measurement of P^{32} of the acid-insoluble phosphate compounds was carried out by the method reported in the previous paper *(19)*.

RESULTS

The amounts of acid-soluble and acid-insoluble phosphate compounds in each cellular component of rat liver, fractionated by Schneider's methods *(4)* are presented in Table I. The total content and concentration (P/N) of acid-soluble inorganic P, $\Delta 7$P, organic P other than $\Delta 7$P are the highest in the S-fr and the lowest in N-fr. As for the acid-insoluble P compounds, the contents of lipid and RNA were highest in the S-fr and lowest in the N-fr. DNA of the homogenate was nearly quantitatively recovered in the N-fr.

The specific activities of acid-soluble phosphate compounds of various cellular fractions wese compared at various time intervals after the injection of P^{32} as shown in Figs. 1–3.

Fig. 1 indicates that, 15–30 minutes after the injection of P^{32}, when the specific activity of inorganic P^{32} of blood was extremely high, the specific activities of inorganic P of the N-fr and Mt-fr showed relatively high values, but they were somewhat lower than that of inorganic P of the S-fr, and 2 hours after the injection no difference was observed among the specific activities of these three fractions. As for the specific activity of the acid-soluble $\Delta 7$P, those of the Mt-fr and N-fr were somewhat lower than that of the S-fr until one hour after the injection of P^{32} (Fig. 2). To examine in detail the results obtained above, the specific activities of $\Delta 7$P of the purified ATP-ADP fraction and of inorganic P of the inorganic P fraction of the mitochondrial fraction were compared with that of the whole homogenate at various time intervals after the injection of P^{31}. As shown in Table 2, the specific activities of $\Delta 7$P and inorganic P of these purified fractions of Mt-fr showed a lower value as compared with that of the whole homogenate at the early stages, comfirming the results described above. With respect to the specific activity of acid-soluble organic P other than $\Delta 7$P, it was remarkable that the specific activity of the Mt-fr showed a considerably lower value than those of the N-fr and S-fr, until (at least) two hours after the injection of P^{32}. (Fig. 3)

The time course of the specific activities of acid-insoluble phosphate compounds are shown in Fig. 4. As can be seen from Fig. 4–1, the specific acitivities of lipid P showed the highest value in the S-fr, followed by the N-fr, and the lowest value in the Mt-fr until four hours after the injection of P^{32}. As for the turnover rate of RNA-P, the time course in Fig. 4–2 suggest that the specific activity of RNA of the N-fr was very high as compared with those of the S-fr and the Mt-fr, of which the latter showed the lowest value up to the 4th hour after the injection. The renewal rate of the DNA was very slow as shown in Fig. 4–3.

TABLE I

Distribution of Various Phosphate Compounds and Nitrogen in Fractions Isolated from Homogenates of Rat Liver in 0.25M Sucrose

		Homogenate		S-fr		Mt-fr		N-fr	
		P (mg. per 100 g. tissues)	P/N (×1000)	P (mg. per 100 g. tissues)	P/N (×1000)	P (mg. per 100 g. tissues)	P/N (×1000)	P (mg. per 100 g. tissues)	P/N (×1000)
Acid-soluble fraction	Total P	93.7± 7.2*	29.6	73.7±12.6	42.3	14.6±3.0	17.0	4.8±1.8	9.7
	Inorg-P	42.7± 1.7	13.5	34.4± 3.6	19.8	5.7±1.3	6.6	1.7±0.2	3.5
	$\Delta 7$P	10.0± 2.2	3.1	6.8± 1.6	3.9	2.1±0.9	2.4	0.9±0.1	1.8
Acid-insoluble fraction	Lipid-P	123.1±10.4	39.0	62.3± 8.0	35.8	42.4±6.2	49.5	16.4±3.8	33.5
	RNA	99.9± 3.0	31.6	65.9±10.2	38.4	24.7±5.3	28.7	10.2±2.2	20.8
	DNA	23.9± 4.3	7.6					23.1±3.9	48.0
N (mg. per 100 g. tissue)		3160		1740		860		490	

* The standard deviation of the mean.

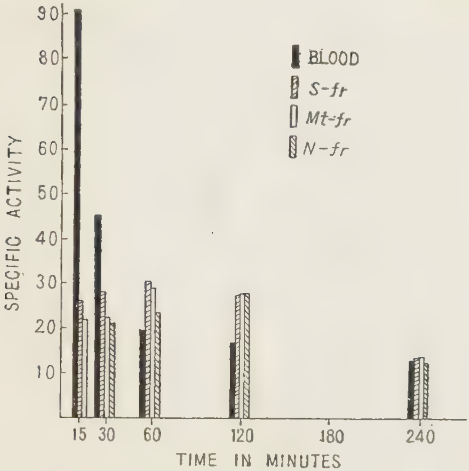

FIG. 1. Specific activities of inorganic P in different rat liver fractions and blood at various time intervals after the intraperitoneal injection of P^{32}. (The value of each period is a mean value of three or four experiments.)

$$\text{Specific activity} = \frac{\text{counts per minutes (c.p.m.)}}{\text{mg. P}} \times \frac{1000}{\text{c.p.m. of injected } P^{32}}$$

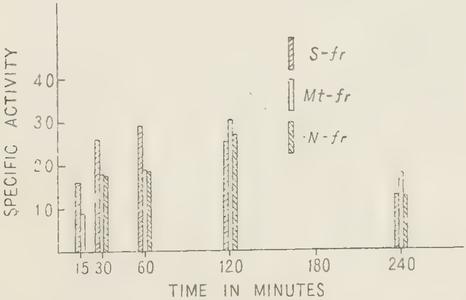

FIG. 2. Specific activities of acid-soluble labile P in different rat liver fractions at various time intervals after the intraperitoneal injection of P^{32}. (The value of each period is the mean value of three or four experiments.)

$$\text{Specific activity} = \frac{\text{c.p.m.}}{\text{mg. p}} \times \frac{1000}{\text{c.p.m. of injected } P^{32}}$$

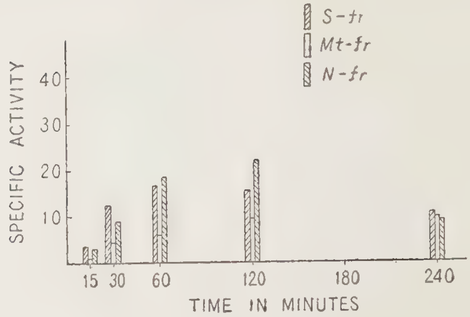

FIG. 3. Specific activities of acid-soluble organic P other than Δ7P in different rat liver fractions at various time intervals after the injection of P^{32}. (The value of each period is the mean of three or four experiments.)

$$\text{Specific activity} = \frac{\text{c.p.m.}}{\text{mg. P}} \times \frac{1000}{\text{c.p.m. injected } P^{32}}$$

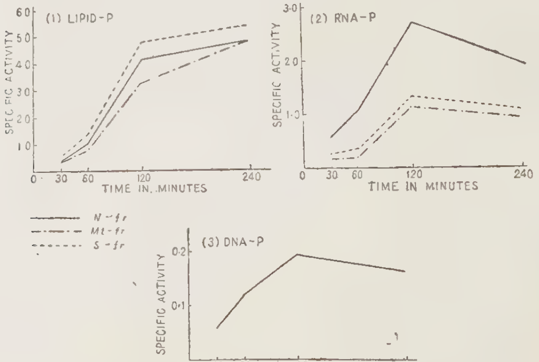

FIG. 4. Specific activities of acid insoluble phosphate compounds in different rat liver fractions at various time intervals after the injection of P^{32}. (The value of each period is the mean of three or four experiments.)

$$\text{Specific activity} = \frac{\text{c.p.m.}}{\text{mg. P}} \times \frac{1000}{\text{c.p.m. injected } P^{32}}$$

DISCUSSION

The use of P^{32} in the tracer experiments affords an oppotunity of learning in more details about the biochemical pathways, by which inorganic P is incorporated into various cellular phosphate compounds. Especially, by employing the differential centrifugation method together with the use of P^{32} it may be possible to demonstrate the exchange rates of phosphate compounds among the various intracellular fractions.

The data shown in Figs. 1 and 2, suggest that the rapid exchanges of inorganic P and $\Delta 7P$ may take place between each of the cellular fractions. As for the rate of renewal of $\Delta 7P$, it may reasonably be expected from the enzymatic experiments of many workers that it would be rather rapid in the Mt-fr. Contrary to such expectation, the Mt-fr showed rather a slow rate of renewal as compared with that of the S-fr and this result was further confirmed by the comparison of $\Delta 7P$ of ATP-ADP fraction of the Mt-fr with that of the whole homogenate, as shown in Table II. This result cannot be explained satisfactorily but it may suggest about the mechanism of the transportation of P^{32} from blood plasma across the cell membrane into the liver cells. With respect

TABLE II

Comparison of the Specific Activities of Labile P of the ATP-ADP Fraction and Inorganic P of the Inorganic P Fraction of the Mt-fr, with Those of the Whole Homogenate at Various Time Intervals after the Injection of Inorganic P^{32}

(The Specific activity of inorganic P of homogenates at each period is expressed as 100)

	Homogenate		Mitochondria	
Time after the injection of P^{32} (*minutes*)	Inorganic P in the inorganic P fraction	$\Delta 7P$ in the ATP ADP fraction	Inorganic P in the inorganic P fraction	$\Delta 7P$ in the ATP-ADP-fraction
15	100 100	39 56	79 59	31 45
30	100 100	92 89	96 70	62 72
60	100 100	115 122	100 81	65 58
120	100 100	78 65	100 92	99 59
240	100 100	78 92	129 95	102 114

to this problem, Sacks (*24*) found plasma inorganic P as a suitable precursor of the labile P of ATP-ADP and glucose-1-phosphate, and assumed that the transportation mechanism of inorganic P may involve the formation of organic phosphate compounds on the cell surface. Recently, *in vitro* studies on the uptake of inorganic P^{32} by human erythrocytes, Gouley (*25*) has presented data which are consistent with the hypothesis that phosphate enters into erythrocyte by the formation of ATP on the cell mambrane, but he has not excluded the possibility that some inorganic P may diffuse into the cells. In connection with the present experimental results that the inorganic P and $\varDelta$7P of the Mt-fr appeared lower than those of the S-fr in the early stage after the injection of P^{32}, it must be mentioned that Lipmann (*26*) has recently suggested that a diffusion barrier for inorganic P exists on the surface of the mitochondria.

As for the time course of the specific activity of lipid P, the present results suggest that there is some relationship between the rate of renewal of this compound and that of the acid-soluble phosphate compounds. Recently, Kornberg and Pricer (*27*) showed that glycerol phosphate may be the intermediate in the formation of phosphate-containing lipids.

There have been several studies made on the renewal rate of RNA in the cellular fractions. Marshak (*14*), Barnum (*16*), and Jenner and Szafarz (*15*) measured the specific activity of nuclear and cytoplasmic RNA in rat liver and mouse embryo after the injection of inorganic P^{32} and found that the specific activity of nuclear RNA was much higher than that of other fractions. Jenner (*15*) proposed a hypothesis from these results that RNA was first synthesized in the nucleus, then migrate to the cytoplasma. However, from the time curve study of the incorporation of P^{32} in RNA in various cellular fractions of mouse liver, Barrum (*16*) considered that the nuclear RNA was less likely to be the precursor of the cytoplasmic RNA. The extremely rapid turnover rate of RNA in the N-fr compared with that in other cellular fractions, as shown in the present paper, suggests that RNA of the nucleus may play somewhat specific role in the metabolism of RNA in the cell.

From the results obtained in the present experiments, it is expected that the soluble fraction including microsomes must be the active site of cellular metabolism. This is in agreement with the data published by Hultin (*17*), which showed that in the liver of newly hatched chicks N^{15}-labeled glycine was incorporated *in vivo* earlier and at a more rapid rate into both protein of microsomes and the supernatant fractions than into the protein of the nuclear and mitochondrial fractions.

SUMMARY

Mitochondrial, nuclear, and soluble fractions (including microsomes) were separated from rat liver tissues, employing Schneider's centrifugal fractionation method. The renewal rates of both acid-soluble and-insoluble phosphate compounds in these fractions were followed at various time intervals after the intraperitoneal injection of P^{32} labeled phosphate. Following results were obtained:

1. Rapid exchange of both inorganic P and acid-soluble labile P was observed among cellular fractions. In early stages after the injection, however, the specific activities of inorganic P and labile P in the soluble fraction (S-fr) were somewhat higher than those of the mitochondrial (Mt-fr) and nuclear fractions (N-fr). These results were further confirmed by comparing the specific activities of inorganic P in the inorganic P fraction, and labile P in the ATP-ADP fraction of the mitochondria with those of the whole homogenate.
2. In the mitochondrial fraction the specific activity of the acid-soluble organic phosphate compounds except for labile P was lower than those in S-fr and N-fr up to the 4th hour after the P^{32} injection.
3. The specific activity of lipid phosphate was higher in S-fr, N-fr, M-fr, in order up to the 4th hour after the injection.
4. Incorporation of P into the nuclear RNA was extremely rapid as compared with that into RNA in S-fr and M-fr; and that into the mitochondrial RNA was slowest.

REFERENCES

(*1*) Claude, A., *J. Exptl. Med.*, **84**, 51 (1946)
(*2*) Schneider, W. C., *J. Biol. Chem.*, **165**, 585 (1946)
(*3*) Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, **172**, 619 (1948)
(*4*) Schneider, W. C., *J. Biol. Chem.*, **176**, 259 (1948)
(*5*) Green, D. E., Loomis, W. F., and Auerbach, V., *J. Biol. Chem.*, **172**, 389 (1948)
(*6*) Lehninger, A. L., and Kennedy, E. P., *J. Biol. Chem.*, **172**, 847, (1948), **179**, 957 (1949)
(*7*) Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **177**, 839

(1949)
(*8*) Cross, R., Covo, G., Taggart, J., and Green, D. E., *J. Biol. Chem.*, **172**, 847 (1948)
(*9*) Lehninger, A. L., *J. Biol. Chem.*, **178**, 625 (1949)
(*10*) Morris, F., and Lehninger, A. L., *J. Biol. Chem.*, **178**, 611 (1949)
(*11*) Graffin, A. L., and Green, D. E., *J. Biol. Chem.*, **176**, 95 (1948)
(*12*) Kennedy, E. P., and Lehninger, A. L., *J. Biol. Chem.*, **172**, 847 (1948); **179**, 957 (1949); **185**, 275 (1950)
(*13*) Dounce, A., *J. Biol. Chem.*, **147**, 685 (1943)
(*14*) Marshak, A. J., *J. Cell. Comp. Physiol.*, **32**, 381 (1948)
(*15*) Jenner, R., and Szafara, D., *Arch. Biochem.*, **26**, 54 (1950)
(*16*) Barnum, C. P., and Huseby, R. A., *Arch. Biochem.*, **29**, 7 (1950)
(*17*) Hultin, T., *Exptl. Cell. Research.*, **1**, 37 (1950)
(*18*) Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936)
(*19*) Ogata, K., Shimizu, T., and Enoki, C., *J. Biochem.*, **40**, 141 (1953)
(*20*) Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945)
(*21*) Schmidt, G., and Thanhauser, S. H., *J. Biol. Chem.*, **161**, 83 (1945)
(*22*) Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925)
(*23*) Enner, A. H., and Rosenberg, H., *Biochem. J.*, **50**, 524 (1951)
(*24*) Sacks, J., *Arch. Biochem. Biophys.*, **30**, 423 (1951)
(*25*) Gouley, D.R.H., *Arch. Biochem. Biophys.*, **40**, 1 (1953)
(*26*) Crane, R. K., and Lipmann, F., *J. Biol. Chem.*, **201**, 245 (1953)
(*27*) Kornberg, A., Pricer, W. E., *Federation Proc.*, **11**, 242 (1952)

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ON THE MECHANISM OF THE ACTIVATION OF α-CHYMOTRYPSINOGEN TO α-CHYMOTRYPSIN

I. ELECTROPHORETIC STUDIES OF THE ACTIVATION OF α-CHYMOTRYPSINOGEN TO α-CHYMOTRYPSIN

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It is an important problem in biochemistry to elucidate the enzymatic activity of protein in its structural terms. Some information about this problem could be obtained by studying the mechanism of the activation of zymogen to active enzyme.

The activation of α-chymotrypsinogen to α-chymotrypsin, a well-known reaction of this type, has been regarded as an enzymatic stepwise modification of the zymogen, because it is mediated by trypsin and presumably accompanied by spontaneous or autocatalytic hydrolysis of intermediate proteins, with the increase of non-protein nitrogen (*1*, *2*, *3*).

The present paper describes the results obtained by electophoretic studies on the activation of α-chymotrypsinogen to α-chymotrypsin.

EXPERIMENTAL

α-Chymotrypsinogen—α-Chymotrypsinogen was prepared from fresh beef pancreas by the method of Kunitz *et al.* (*4*). It was crystallized four times with ammonium sulfate, and then once with aqueous ethanol. One mg. of the final crystals contained 0.142 mg. protein nitrogen.

Trypsin—Crystalline trypsin supplied from the Armour and Company, Chicago, Illinois, U.S.A., was employed; 1 mg. of the crystals contained 0.093 mg. protein nitrogen, and its specific activity was 0.23 $[\mathrm{T.U.}]^{\mathrm{cas.}}_{\mathrm{mg.P.N.}}$

Soybean Trypsin Inhibitor—Crystalline soybean trypsin inhibitor was prepared from defatted soybean by the method of Kunitz *et al.* (*4*).

Activation of α-Chymotrypsinogen—Crystalline α-Chymotrypsinogen (372 mg.) was suspended in 8 ml. $M/2$ Søensen phosphate buffer (pH 7.6) and dissolved by the addition of one or more drops of 5 N sulfuric acid, and sodium hydroxide solution equivalent to the acid was also added. The mixture was diluted with distilled water to 32 ml., cooled to 5°, and activated at the same temperature by the addition of 0.85 mg. of crystalline trypsin dissolved in 0.5 ml. 0.0025 M hydrochloric acid. Aliquots were removed at various time intervals and their proteolytic activities were determined. At the same time, the solutions were studied electrophoretically after the termination of the tryptic activity by adding soybean trypsin inhibitor.

Determination of Proteolytic Activity—The activation mixture (0.1 ml.) was diluted with water to an adequate enzyme concentration for the determination of proteolytic activity. Then, 1 ml. of the solution was added to 1 ml. casein solution (1 per cent), mixed well, and incubated at 35° for 20 minutes. The solution was then poured back and forth into a tube containing 3 ml. of 5 per cent tridloracetic acid and the precipitate was filtered after standing one hour or longer at about 25°. The concentration of tyrosine in the filtrate was determined electrophotometrically as usual (*4*). The proteolytic activity was calculated by the following equation, and shown in Fig. 1.

$$[\text{Xt-U}]^{\text{cas.}}_{\text{ml.}} = \frac{\text{milliequivalents of soluble tyrosine}}{\text{incubation time (in minutes)} \times \text{ml. of activation mixture}}$$

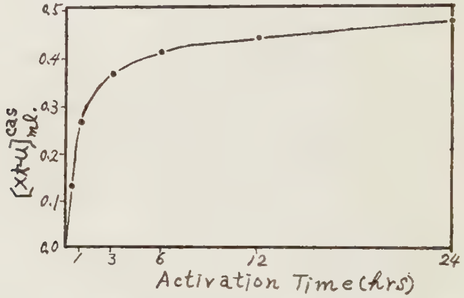

FIG. 1. The proteolytic activity during the activation process.

Electrophoretic Study of the Activation Process—The electrophoretic experiment was carried out as follows. In order to terminate the tryptic activity, 0.5 ml. soybean trypsin inhibitor solution (1.91 mg. crystalline inhibitor was dissolved in 15.9 ml. of 0.0025 M hydrochloric acid) was added to 3.5 ml. of the activation mixture. The solution was then adjusted with 5 N sulfuric acid to a pH 3.7,[1] and dialyzed at 2° for 48 hours in cellophane bag against 500 ml. of pH 3.62 acetate buffer (ionic strength, 0.1). Electrophoresis was carried out in a 50×15×2 mm. cell.

Fig. 2 represents the electrophoretic patterns[2] during the activation process, and Table I shows the mobilities of the components. Calculations were made from the ascending boundary, because the electrophoretic patterns in the descending boundary were remarkably spread for every components.

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[1] At this pH, the autolysis of α-chymotrypsin are negligibly small.

[2] Neither trypsin nor soybean trypsin inhibitor comes out in the electrophoretic patterns, because of their small refractive increment.

TABLE I

Components and their Mobilities during the Activation Process*

Activation time \ Components	A	B	C_1	C_2	C_3	D_1	D_2	D_3	E_1	E_2	F	G_1	G_2
0 *hrs.*	5.49												
1/2	5.48			4.62	4.33						1.96		0.94
1	5.49			4.62						2.38			
3	5.48	5.14					3.58						
6	5.48	5.13	4.81	4.62				3.22	2.66				
12	5.48		4.81										
24	5.48					3.64						0.98	

* $\times 10^{-5}$ cm^2/volt. sec.

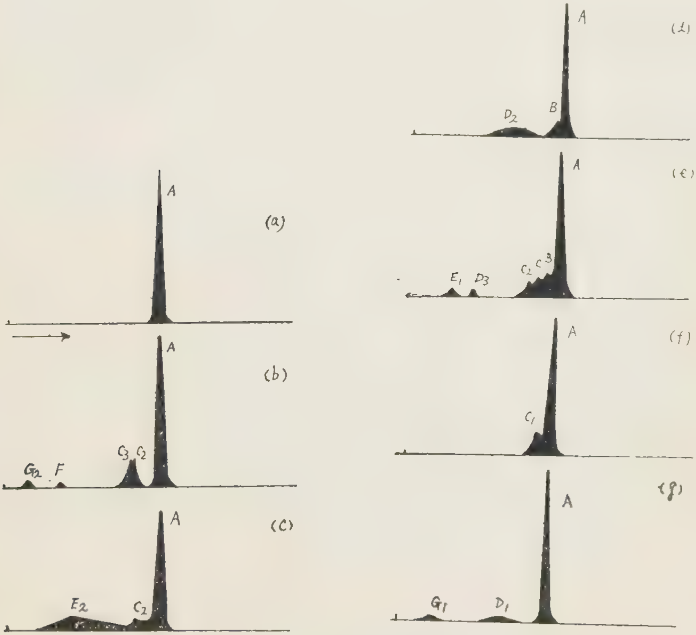

FIG. 2. Electrophoretic Patterns during the activation process. (a), (b), (c), (d), (e), (f), and (g) are electrophoretic patterns of the activation mixtures which were activated for 0, 1/2, 1, 3, 6, 12 and 24 hours respectively. (in an 0.1 *M* acetate buffer at pH 3.62, after 5922 sec. at 8.2 volt/cm.).

DISCUSSION

The activation of *α*-chymotrypsinogen by trypsin has been recognized to be a very complicated process, proceeding through two intermediates, called *π*-chymotrypsin and *δ*-chymotrypsin, and yielding a mixture of several chymotrypsins designated by the letters *α*, *β* and *γ* (*1*, *3*).

In the present experiment, therefore, attempt was made to activate *α*-chymotrypsinogen so as to lead mainly to *α*-chymotrypsin (*1*), but the results obtained indicated that this process was so complicated that no complete interpretation about the mechanism of the activation was possible except the following points.

1) Fig. 1 shows that *α*-chymotrypsinogen is activated to about 80 per cent in 6 hours, and main component A in Fig. 2 must be regarded as a mixture of the zymogen and the active enzyme. Therefore, *α*-chymotrypsinogen seems to have the same mobility as *α*-chymotrypsin in the experimental conditions. Experiments carried out in the other pH for the purpose of seperating the two components were not successful.

2) Even when it was activated for half an hour, four new homogeneous components, C_2, C_3 with large mobilities and F, G with small mobilities already appeared, but after half an hour these four components disappeared and another two inhomogeneous components appeared. Thus in all, 12 components B-G_2 revealed themselves during the activation process for 24 hours.

3) As small polypeptides liberated during the activation process had been dialyzed away before electrophoresis, many components ascertained in the electrophoresis might be intermediatdes or liberated polypeptides of relatively high molecular weights.

4) It could be concluded from the above described results, that the activation process of *α*-chymotrypsinogen to *α*-chymotrypsin must not be a simple transformation as previously supposed but involves a highly complex one.

SUMMARY

1. The activation of *α*-chymotrypsinogen by trypsin has been studied electrophoretically and the appearance of many components of relatively high molecular weight were confimred in the course of the activation.

2. The activation of α-chymotrypsinogen to α-chymotrypsin seems to be a process of high complexity accompanied by the formation of a number of relatively large molecular degradation products or intermediates.

The author wishes to thank Professor Shiro Akabori for his guidance throughout of this work, also Shuichiro Kubo for his valuable technical assistance.

REFERENCES

(*1*) Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, **22**, 207 (1938)
(*2*) Butler, J.A.V., *J. Am. Chem. Soc.*, **63**, 2968 (1941)
(*3*) Jacobsen, C. F., *Compt. rend. trav. lab. Carlsberg, Sér. Chim.*, **25**, 325 (1947)
(*4*) Northrop, J. H., Kunitz, M., and Herriott, M., Crystalline enzymes, Columbia Univ. Press, New York, 2nd edition (1948)

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STUDIES ON TAKA-AMYLASE A.*

II. AMINO ACID COMPOSION OF TAKA-AMYLASE A

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(Received for publication, September 25, 1954)

In an enzyme which contains no characteristic active group other than usual peptide chain, the structural responsibility for the enzymatic action must be attributed to some particular petptide part of a specific amino acid sequence. For the determination of amino acid sequence in proteins or peptides, it is primarily necessary to have exact informations about their amino acid composition.

All amylases, heretofore obtained in crystalline state, are regarded to belong to simple protein bearing no prosthetic group. The complete analysis of pancreatic amylase has been recently reported by Caldwell *et al.* (*1*), but on other amylases no detailed analytical data on the amino acid composition are available.

As reported in the previous papers (*2*, *3*) the authors were able to obtain sufficient quantity of crystalline taka-amylase A (α-type) by a relatively simple method, and therefore, the complete analysis of this enzyme protein was undertaken.

The results of the quantitative determination of all amino acids based on the chromatographic, colorimetric and enzymatic methods are described in this paper.

EXPERIMENTS AND RESULTS

Materials—Crystalline taka-amylase A obtained from synthetic culture media (*2*) or " Takadiastase Sankyo " (*3*) was recrystallized three times from aqueous acetone, and washed with 50 per cent acetone and then with acetone, dried in a vacuum desiccator. Results of the elemental analysis of a specimen after being dried over P_2O_5 to a constant weight at 56° in vacuum, are as follows:

C 48.10 per cent, H 7.47 per cent, N 14.98 per cent, S 0.68 per cent, Ca 0.72

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* Comm. I appeared in *J. Biochem.*, **41**, 577 (1954)

per cent.

Sugar content 2.75 per cent.

Hydrolysis—Crystalline taka-amylase A was hydrolyzed with 15 times the weight of 6 *N* HCl in a sealed glass tube for 24 hours at 100°. After removal of the excess of hydrochloric acid under reduced pressure, the residual hydrolysate was taken up in a definite volume of proper solvent and used for analysis.

Colorimetric and Enzymatic Determination of Individual Amino Acids—Methods employed for colorimetric analysis of individual amino acids are listed in Table I.

TABLE I

Amino acid	Method of analysis
Phenylalanine	Kapellar-Adlar's reaction (*4–7*)
Arginine	Sakaguchi's reaction (*8*)
Tyrosine	Millon's reaction (*9–12*)
Histidine	Hunter's reaction (*13*)
Cystine	Phospho-tungstate method (*14*)
Tryptophan	Spies, Chambers's method (*15, 16*)

Glutamic acid content was determined by enzymatic decarboxylation by measuring CO_2 evolution, Warburg's apparatus being employed. Glutamic decarboxylase used in the determination was prepared from squash according to Okunuki and Inagaki (*17*). This enzyme preparation contains not only L-glutamic decarboxylase but also transaminase, α- and β-carboxylase in appreciable amount. Aspartic acid could, therefore, be determined manometrically by the use of the same enzyme preparation according to Okunuki, Sakurai and Matsuda (*18*). In the presence of excess of α-ketoglutaric acid, 3 moles of CO_2 are evolved for 1 mole of aspartic acid as follows.

$$\begin{tikzcd}
\text{Aspartic acid}+\alpha\text{-ketoglutaric acid} \arrow[d, "\text{Transaminase}"] & \\
\text{Oxalacetic acid}+\text{glutamic acid} \arrow[d, "\alpha\text{- and }\beta\text{-Carboxylases}"] \arrow[dr, "\text{L-Glutamic decarboxylase}"] & \\
\text{Acetaldehyde}+2CO_2 & \gamma\text{-Amino butyric acid}+CO_2.
\end{tikzcd}$$

Results of the colorimetric analyses of six amino acids listed in Table I and the results of enzymatic determination of aspartic and glutamic acid are shown in Table II.

Systematic Chromatographic Analysis—Chromatographic determinations of amino acids were carried out by Stein and Moore's technique using starch column and following two solvent systems successively (*19–22*).

1) *n*-Butanol-*n*-prophanol-0.1 *N* HCl (1:2:1)
2) *n*-Propanol-0.5 *N* HCl (2:1)

The distribution of amino acids in effluent was similar to that reported by Stein and Moore (*21*). By means of the solvent systems used in the above experiments amino acids belonging to the following three groups could not be separated to single ones:

Group A: Leucine, isoleucine and phenylalanine.
Group B: Tyrosine, valine and methionine.
Group C: Glutamic acid and alanine.

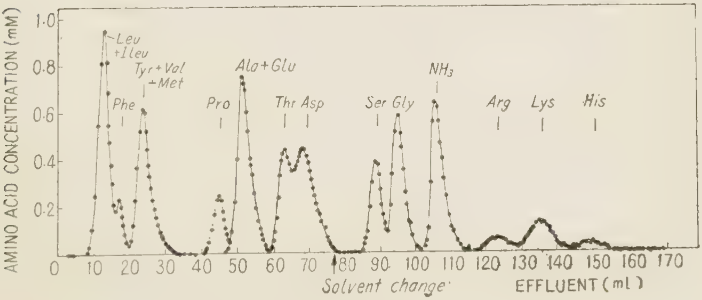
TABLE II

Amino Acid Composition of Taka-amylase A

Amino acid	g. of amino acid per 100 g. protein	g. of amino acid residue per 100 g. of protein	N as per cent of protein N
Tryptophan*	3.87	3.53	3.54
Penylalanine	5.98	5.34	3.39
„ **	5.81	5.18	3.29
Arginine	4.68	4.21	10.1
Tyrosine	11.77	10.60	6.06
„ **	11.91	10.75	6.15
Histidine	1.43	1.26	2.58
Cystine+cysteine	1.6	1.37	1.24
Glutamic acid	6.81	5.98	4.32
Aspartic acid	9.52	8.23	6.71

* Tryptophan was determined directly on the enzyme protein without hydrolysis.
** Analysis with alkali hydrolysate.

Fig. 1



Contents of these groups were calculated, therefore, assuming the factors of colour yield 1.00, 1.00 and 1.05 respectively, as compared to the colour yield of leucine 1.00. The results of the analysis are represented in Fig. 1 and the numerical values are shown in Table III.

The separation and determination of phenylalanine, leucine, isoleucine, me-

TABLE III

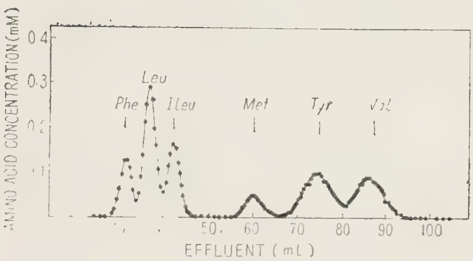
Chromatographic Analysis of Hydrolysates of Taka-amylase A

Solvent: *n*-Butanol-*n*-propanol-0.1 *N* HCl (1:2:1)
and *n*-propanol-0.5 *N* HCl (2:1)
Starch column, 0.9×30 cm. Sample, 2.0–2.5 mg.

Chromatogram No.	8		9		Average
Amino acid	g. of amino acid per 100 g. of protein	N as per cent of protein N	g. of amino acid per 100 g. of protein	N as per cent of protein N	N as per cent of protein N
Leucine Isoleucine Phenylalanine		10.98		11.51	11.25
Valine Tyrosine Methionine		10.40		10.80	10.60
Proline	6.08	4.93	4.10	3.33	3.33*
Glutamic acid Alanine		11.18		11.08	11.13
Threonine		18.62	9.95	7.79	7.79
Aspartic acid			16.22	11.42	11.42
Serine	6.02	5.34	6.32	5.62	5.48
Glycine	6.62	8.24	6.32	7.88	8.06
Ammonia	1.62	8.89	1.84	10.07	9.48
Arginine	3.36	7.20	3.44	7.40	7.30
Lysine	5.71	7.30	6.16	7.90	7.60
Histidine	1.91	3.46	1.89	3.39	3.43
Cystine+cysteine	—	—	—	—	—
Totals		96.54		97.89	97.27

* This figure is the value for chromatogram 9, as value of chromatogram 8 for proline is not available, because the separation was incomplete.

Fig. 2



thionine, tyrosine and valine were achieved by the use of *n*-butanol-benzyl alcohol-water (1:1: 0.284) as solvent. The results are shown in Fig. 2 and Table IV.

Stein and Moore (*19*) reported that methionine was oxidized with a trace of peroxide in benzyl alcohol, while the oxidation was inhibited completely by the ad-

TABLE IV

Chromatographic Analysis of Hydrolysates of Taka-amylase A and Taka-amylase from Synthetic Culture Solution

Solvent: *n*-Butanol-benzyl alcohol-water-thiodiglycol
(1:1:0.284: 0.01)
(chromatogram 10 is thiodiglycol-free)
Starch column, 0.9×30 cm. Sample, 2.0–3.0 mg.

	Taka-amylase A from " Takadiastase Sankyo "					Taka-amylase from culture	
Chromatogram No.	10		22		Average	21	
Amino Acid	g. of amino acid per 100 g. of protein	N as per cent of protein N	g. of amino acid per 100 g. of protein	N as per cent of protein N	N as per cent of protein N	g. of amino acid per 100 g. of protein	N as per cent of protein N
Phenylalanine	4.19	2.33	4.30	2.39	2.36	4.05	2.25
Leucine	8.30	5.80	8.29	5.79	5.80	8.07	5.63
Isoleucine	5.38	3.76	5.00	3.49	3.63	5.14	3.59
Methionine	2.10	1.29	2.20	1.35	1.35	2.32	1.43
Tyrosine	9.38	4.75	9.75	4.93	4.84	10.00	5.07
Valine	4.28	3.35	5.09	3.98	3.67	4.70	3.67

dition of 0.5 per cent thiodiglycol to the solvent. In the authors' experiments, however, there was no difference in the amounts of methionine recovered between the solvents with and without thiodiglycol.

Jensen (*24*) pointed out that there are unavoidable large errors in analytical data for basic amino acids in starch chromatography, and therefore, three basic amino acids, lysine, arginine and histidine were determined by means of ion-exchange chromatography according to Stein and Moore (*23*). The column was made up with Na form of Amberlite IR-120 of 150 mesh. The buffer solution was prepared

Fig. 3

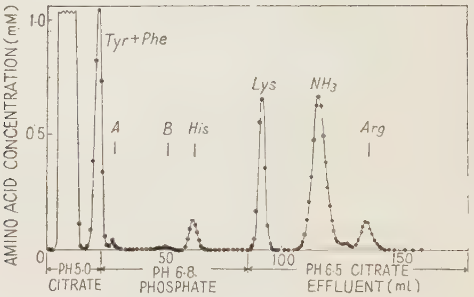


TABLE V

Chromatographic Analysis of Basic Amino Acids of Taka-amylase A

Buffer solution (*23*); pH 5.0 citrate buffer, pH 6.8 phosphate buffer, pH 6.5 citrate buffer
Column: Amberlite IR-120 Na form, 0.9 × 15 cm.

Chromatogram No.	12		14		Average
Amino acid	g. of amino acid per 100 g. of protein	N as per cent of protein N	g. of amino acid per 100 g. of protein	N as per cent of protein N	N as per cent of protein N
Histidine	1.98	3.57	1.98	3.56	3.57
Lysine	5.93	7.59	5.70	7.29	7.44
Ammonia	1.63	8.95	1.78	9.77	9.36
Arginine	2.43	5.21	2.91	6.24	5.73
Tyrosine Phenylalanine		} 7.12		} 7.09	} 7.11

by the direction given by Stein and Moore, except that no versenate (ethylendiaminetetraacetic acid disodium salt) and no detergent were added. The chromatography was carried out at room temperature and it was observed that there was no significant difference in the results due to the difference in temperature between 10–25°.

TABLE VI

Amino Acid Composition of Taka-amylase A

Amino acid	g. of amino acid per 100 g. of protein	g. of amino acid residue per 100 g. of protein	N as per cent of protein N	moles of amino acid per 10^5 g. of protein
Leucine	8.30	7.17	5.80	63.3
Isoleucine	5.20	4.49	3.63	39.7
Phenylalanine	4.25	3.78	2.36	25.8
Tyrosine	9.55	8.60	4.84	52.8
Valine	4.69	3.97	3.67	40.1
Methionine	2.20	1.94	1.35	14.8
Alanine	6.80	5.43	6.98	76.3
Glutamic acid	6.95	6.10	4.32	47.2
Threonine*	10.68	9.08	8.20	85.3
Aspartic acid	16.53		11.42	
Aspartic acid (free)		4.18		36.4
Asparagine		10.08		88.4
Serine*	6.48	5.37	6.09	61.6
Glycine	6.59	5.01	8.06	87.8
Amide NH_3*, **	1.50		8.10	
Arginine	2.72	2.45	5.73	15.7
Lysine	5.94	5.19	7.44	40.7
Histidine	2.02	1.78	3.57	13.0
Cystine+cysteine	1.60	1.37	1.24	6.7
Tryptophan	3.97	3.60	3.54	19.2
Proline	4.18	3.52	3.33	33.0
Totals	110.15	93.11	99.69	847.8

* The values of serine and threonine were corrected by 10 per cent and 5 per cent respectively for the destruction during acid hydrolysis of protein, and the value of ammonia was correspondingly reduced according to Rees (*25*).

** This value is in a good agreement with the value obtained by a separate determination of usual amide N analysis.

The results of the analysis of basic amino acids are given in Fig. 3 and Table V.

Amino Acid Composition of Taka-amylase A—All of the results of analyses described above are summarized in Table VI.

DISCUSSION

Taka-amylase A protein distinguishes itself from usual proteins by its low contents of cystine and methionine, relatively high contents of tyrosine and threonine and that the ratio, aspartic acid/glutamic acid, is as high as 2.6.

There are distinct differences between the results of elemental analyses reported by Fischer *et al.* (*26*), by Caldwell *et al.* (*27*) and by the present authors. These differences might arise from the difference of purity between taka-amylase preparations used for analysis by the different authors, from the difference of strain of *Aspargillus oryzae*, from which taka-amylase was produced, or from the difference of conditions of culture.

N-recovery in the analysis is as high as 99.69 per cent while the sum of amino acid residues is 93.11 per cent of ash free protein. This

Table VII

Minimum Molecular Wweight of Taka-Amylase A

Amino acid	Molar ratio of amino acid against 7 moles histidine		Minimum molecular weight
Leucine	34.2	34	53,700
Isoleucine	21.4	21	52,900
Phenylalanine	13.9	14	54,300
Tryosine	28.4	28	53,000
Valine	21.6	22	54,800
Methionine	8.0	8	54,000
Alanine	41.2	41	53,700
Glutamic acid	25.4	25	53,000
Aspartic acid	19.7	20	55,000
Asparagine	47.7	48	54,300
Glycine	47.4	47	53,600
Arginine	8.5	9	57,400
Lysine	22.0	22	54,100
Histidine	7.0	7	53,800
Tryptophan	10.4	10	52,100
Proline	17.8	18	54,500
Cystine	3.6	4	59,800
Serine	33.1	33	53,500
Threonine	45.9	46	53,800
			54,000±700

fact is probably due to the destruction of tryptophan and tyrosine during the acid hydrolysis and the presence of carbohydrate in the enzyme protein. As it was already demonstrated by Yoritake *et al.* (*28*) and Akabori *et al.* (*29*, *30*) the destruction of tryptophan and tyrosine is greatly accelerated by the presence of carbohydrate and other carbonyl compounds, and small quantity of alanine and serine are thereby produced.

Two small peaks A and B in Fig. 3 were not yet identified, but from their position they can be assumed to be β-alanine and tryptophan respectively, a small quantity of the latter probably remains in the hydrolysate, because the content of this amino acid is relatively high in taka-amylase A. The N per cent of A and B to total N are 0.35 and 0.14, respectively.

The molecular weight of taka-amylase A was calculated to be about 53,000 from the data of amino terminal group determination which will be reported in the succeding paper (*31*) and pressure-area relation of surface film of this enzyme protein. The latter experiment was carried out in the laboratory of Prof. Isemura of the Institute of Scientific and Industrial Research, Osaka University.

In the Table VII, the numbers of amino acid residues in one mole of taka-amylase A are given assuming the molecular weight of the enzyme as 54,000±700 which is the theoretical value derived from the assumption that taka-amylase molecule contains 7 histidine residues.

SUMMARY

The amino acid composition of crystalline taka-amylase A has been determined by colorimetric, enzymatic and chromatographic analyses. The minimum molecular weight of the enzyme is calculated to be 54,000±700.

The authors wish to express their sincere tanks to Sankyo Co., Ltd. for their kind supply of " Takadiastase Sankyo " and to Mr. S. Sakurai for the enzymatic analyses of glutamic and aspartic acids. A part of the expense for this research was defrayed by the Grant for Scientific Research of the Ministry of Education to whom the authors' thanks are due.

REFERENCES

(*1*) Caldwell, M. L., Dickey, E. M., Hanrahan, V. M., Kung, H. C., Kung, J. T., and Misko, M., *J. Am. Chem. Soc.*, **76**, 143 (1954)

(*2*) Akabori, S., Hagihara, B., and Ikenaka, T., *Proc. Japan Acad.*, **27**, 350 (1951)

(*3*) Akabori, S., Ikenaka, T., and Hagihara, B., *J. Biochem.*, **41**, 577 (1954)

(*4*) Jervis, G. A., *J. Biol. Chem.*, **134**, 105 (1940)

(*5*) Jervis, G. A., *J. Biol. Chem.*, **134**, 567 (1940)

(*6*) Block, R. J., and Diana, B., *J. Biol. Chem.*, **129**, 1 (1939)

(*7*) Kappelar-Adler, *Biochem. Z.*, **252**, 185 (1932)

(*8*) Weber, C. J., *J. Biol. Chem.*, **86**, 217 (1930)

(*9*) Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927)

(*10*) Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929)

(*11*) Lugg, J.W.H., *Biochem. J.*, **31**, 1422 (1937)

(*12*) Gran, C. R., *J. Biol. Chem.*, **168**, 485 (1947)

(*13*) Hunter, A., *J. Biol. Chem.*, **196**, 589 (1952)

(*14*) Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 115 (1938)

(*15*) Spies, J. R., and Chambers, D. C., *Anal. Chem.*, **20**, 30 (1948)

(*16*) Spies, J. R., and Chambers, D. C., *Anal. Chem.*, **21**, 1249 (1949)

(*17*) Okunuki, K., and Inagaki, M., *Proc. Japan Acad.*, **27**, 658 (1951)

(*18*) Okunuki, K., Sakurai, S., and Matsuda, G., *Proc. Japan Acad.*, **28**, 347 (1952)

(*19*) Stein, W. H., and Moore, S., *J. Biol. Chem.*, **176**, 337 (1948)

(*20*) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948)

(*21*) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **178**, 53 (1949)

(*22*) Stein, W. H., and Moore, S., *J. Biol. Chem.*, **178**, 79 (1949)

(*23*) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192**, 663 (1951)

(*24*) Jensen, R., *Acta Chem. Scand.*, **6**, 771 (1952)

(*25*) Rees, M. W., *Biochem. J.*, **40**, 632 (1946)

(*26*) Fischer, E. H., and de Montmollin, R., *Helv. Chim. Acta*, **34**, 1994 (1951)

(*27*) Hanrahan, V. M., and Caldwell, M. L., *J. Am. Chem. Soc.*, **75**, 4030 (1953)

(*28*) Yoritaka, T., *Mitt. Med. Ges. Osaka*, **38**, 1183 (1939)

(*29*) Akabori, S., and Narita, K., *Proc. Japan Acad.*, **25**, 6 (1949)

(*30*) Akabori, S., and Ohno, K., *Proc. Japan Acad.*, **26**, 39 (1950)

(*31*) Akabori, S., and Ikenaka, T., *J. Biochem.* in press

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